# *Monocyte and macrophage regulation of pulmonary fibrosis*

Michael A. Gibbons

**PhD Thesis**

**The University of Edinburgh**

**2010**

## ABSTRACT

In this thesis I examined the role of circulating monocytes and lung macrophages in the pathogenesis of the early fibrotic, progressive fibrotic and resolution phases of pulmonary fibrosis.

Pulmonary fibrosis with destruction of lung architecture and consequent respiratory failure and death represents a massive worldwide health burden. Although idiopathic pulmonary fibrosis (IPF) is the archetypal and most common cause of lung fibrosis, numerous respiratory diseases can progress to pulmonary fibrosis, and this usually signifies a worse prognosis. Importantly, the incidence and prevalence of IPF continue to rise and it remains one of the few respiratory conditions for which there are no effective therapies.

The mechanisms resulting in pulmonary fibrosis are controversial. Early work in the 1980s and 1990s suggested that lung macrophages were important. However, at the turn of the 21st century there was a shift to a belief that pulmonary fibrosis resulted from aberrant wound healing as a consequence of repetitive epithelial injury from an as yet unknown cause. However, with the ever expanding knowledge of the importance of macrophages in other fibrotic conditions such as the kidney and liver, the potential importance of macrophages in pulmonary fibrosis has become more pertinent.

Using an *in vivo* depletional strategy in several murine models of lung fibrosis, in conjunction with human studies, I sought to characterise the role of circulating monocytes and lung macrophages in the pathogenesis of pulmonary fibrosis.

I have established that circulating monocytes and lung macrophages are not critical for the development of early lung fibrosis. In contrast, circulating monocytes and lung macrophages are important during the progressive fibrotic phase of lung fibrosis.

Furthermore, my data suggest that the pro-fibrotic alternatively activated macrophages may be the sub-class of macrophages that mediate this fibrogenic effect. In addition and in contrast, I have established that lung macrophages are required for the resolution of fibrosis. This finding is in keeping with important work performed in the field of liver fibrosis.

There is an ever increasing literature examining the role of matrix metalloproteinases (MMPs) during tissue fibrosis and repair. My work has suggested that during lung fibrosis there may be compartmental specific functions of MMPs that regulate lung fibrogenesis, although more work is required before this exciting finding can be properly defined.

## ACKNOWLEDGEMENTS

I am indebted to my supervisors, Professors Stuart Forbes, Tariq Sethi and John Iredale, for excellent guidance, support and advice throughout my PhD. I am particularly indebted to Professor Forbes who has been fabulous throughout the PhD. I would like to thank Dr Alison MacKinnon for helping me learn the practicalities of laboratory work and for being a constant source of discussion, help and advice. I am also grateful to Dr Nikhil Hirani and Professor Sarah Howie for guidance and advice and to Professor Chris Haslett and Dr John Simpson without whom undertaking my PhD would not have been possible. I would like to thank Dr Jeremy Hughes who kindly supplied the CD11b-DTR mice, Professor Chris Gregory who supplied the $CX_3CR1$ mice, and Professor Gunter Hammerling (Deutsches Krebsforschungszentrum (DKFZ, German Cancer Research Centre), Heidelberg) and Dr Andrew MacDonald (Institute of Immunology and Infection Research, University of Edinburgh) who supplied the CD11c-DTR mice. I'd like to thank Dr Kay Samuel for help with flow cytometry, Professor Sarah Howie and Dr William Wallace for help with histological interpretation and scoring, and Fiona Rossi and Dr Prakash Ramachandran for help with flow sorting. I'd like to thank all members of the Forbes, Iredale and Sethi groups, and Kev Dhaliwal, Rodger Duffin and Professor Adriano Rossi of the lung inflammation group, for help and support throught my PhD. Most importantly, I'd like to thank my unique wife Lisa for her unconditional support and understanding throughout my PhD studies, my parents John and Rose, and my brothers John and Kevin, for their patience, support and understanding. Lastly, I'd be like to thank my wonderful children Oscar, Felix and Rosalila who have brought, and continue to bring joy to all aspects of my life.

## DECLARATION

This thesis has been written by myself and represents my own work. All of the experiments described herein were performed by me.

## AIMS & HYPOTHESIS

It is my hypothesis that circulating monocytes and lung macrophages have an important role in the pathogenesis of pulmonary fibrosis. Using *in vivo* depletional, add-back and add-in experiments I wish to establish whether this is indeed the case. I aim to use the above strategies to investigate at which stages during lung fibrogenesis and fibrosis resolution circulating monocytes and lung macrophages may be important. Furthermore, I wish to establish whether potentially pro-fibrotic alternatively activated macrophages play a role in disease pathogenesis, and by focusing on matrix metalloproteinase biology I aim to gain some mechanistic insights. Finally, by using samples from patients with fibrotic lung diseases I aim to establish whether this hypothesis has relevance to the human form of disease.

.

## TABLE OF CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| Ad | Adenoviral |
| AMDGF | Alveolar macrophage-derived growth factor |
| ARDS | Adult respiratory distress syndrome |
| ATS | American Thoracic Society |
| BALF | Bronchoalveolar lavage fluid |
| BM | Basement membrane |
| BMDM | Bone marrow-derived macrophage |
| CTGF | Connective tissue growth factor |
| DMEM | Dulbecco's modified eagle medium |
| DNA | Deoxyribonucleic acid |
| DPLD | Diffuse parenchymal lung disease |
| DT(R) | Diphtheria toxin (receptor) |
| ECM | Extra-cellular matrix |
| ELISA | Enzyme-linked immunosorbent assay |
| EMT | Epithelial mesenchymal transition |
| ERK | Extracellular signal regulated kinase |
| ERS | European Respiratory Society |
| FACS | Fluorescence-activated cell sorting |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| (F)VC | (Forced) vital capacity |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| H&E | Haematoxylin and eosin |
| (HR)CT | (High resolution) computed tomography |
| HRP | Horseradish peroxidase |
| i.p. | intra-peritoneal |
| i.t. | intra-tracheal |

| | |
|---|---|
| ICC | Immunocytochemistry |
| IGF | Insulin-like growth factor |
| IHC | Immunohistochemistry |
| IIP | Idiopathic interstitial pneumonia |
| IL | Interleukin |
| IFNγ | Interferon-γ |
| IPF | Idiopathic pulmonary fibrosis |
| JNK | c-jun N-terminal kinase |
| LC | Liposomal clodronate |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| M-CSF | Macrophage-colony stimulating factor |
| MCP | Monocyte chemotactic protein |
| MHC | Major histocompatiability complex |
| MIP | Macrophage inhibitory protein |
| MMP | Matrix metalloproteinase |
| NO(S) | Nitric oxide (synthase) |
| NSIP | Non-specific interstitial pneumonia |
| OVA | Ovalbumin |
| PCR | Polymerase chain reaction |
| PBS | Phosphate-buffered saline |
| PDGF | Platelet-derived growth factor |
| PPAR | Peroxisome proliferator-activated receptor |
| QoL | Quality of life |
| RNA | Ribonucleic acid |
| RPM | Rotations per minute |
| SAP | Serum amyloid protein |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SLE | Systemic lupus erythematosis |

| | |
|---|---|
| STAT | Signal transducer and activator of transcription |
| TGFβ | Transforming growth factor-β |
| TIMP | Tissue inhibitor of matrix metalloproteinase |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| UIP | Usual interstitial pneumonia |

# CHAPTER 1

# INTRODUCTION

## Fibrotic lung diseases

The human fibrotic lung diseases encompass a variety of specific diseases of different underlying aetiologies. Pulmonary fibrosis affects over 5 million people worldwide (Maher *et al*., 2009) and as such is an enormous health burden. In 2002 the American Thoracic Society (ATS) and European Respiratory Society (ERS) published a consensus classification on the idiopathic interstitial pneumonias (IIPs) which they defined within the family of diffuse parenchymal lung diseases (DPLDs). It is within this family of DPLDs that the various forms of fibrotic lung disease may be found (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias, 2002) (Figure1.1). The causes of fibrotic lung disease include those associated with collagen vascular disease – rheumatoid arthritis, systemic lupus erythematosis (SLE) and systemic sclerosis – the granulomatous lung diseases which may progress to lung fibrosis such as sarcoidosis, chronic hypersensitivity pneumonitis, occupational lung diseases such as asbestosis and silicosis, the adult respiratory distress syndrome (ARDS) and chronic lung infections such as tuberculosis. Within the family of DPLDs the largest subgroup is the IIPs, the commonest of which is idiopathic pulmonary fibrosis (IPF). IPF is the archetypal human fibrotic lung disease.

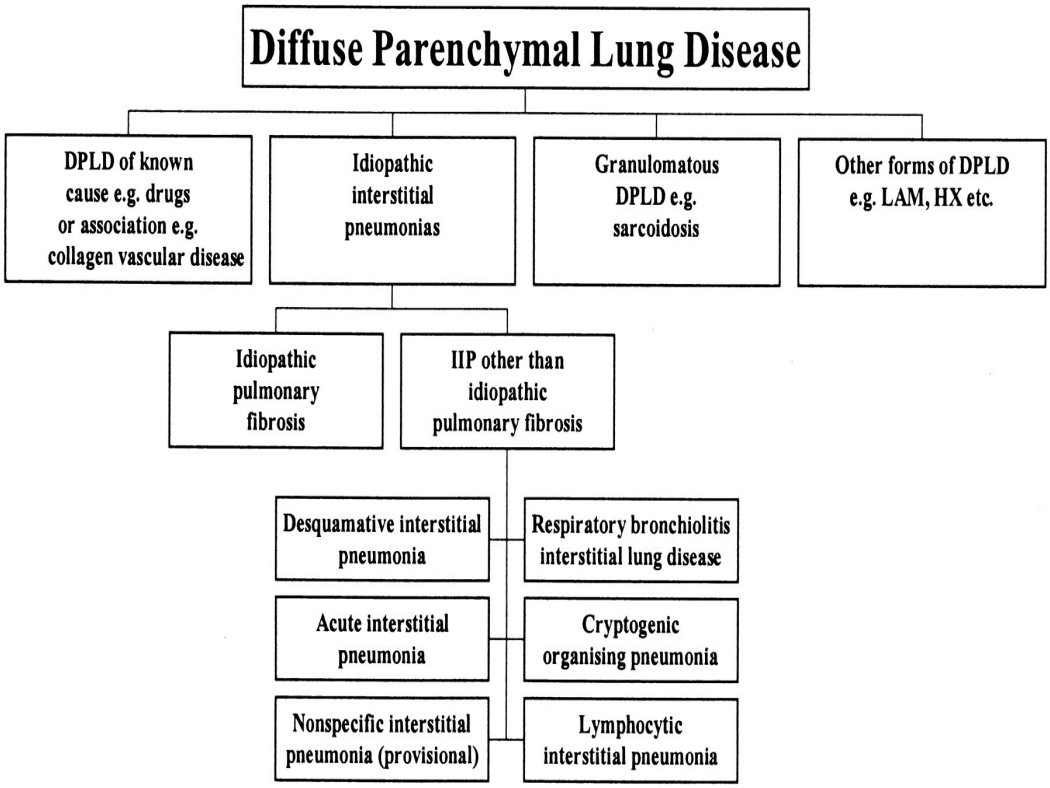



**Figure 1.1. Classification of the diffuse parenchymal lung diseases.**

From: American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med* 2002; 165:277-304.

## Idiopathic Pulmonary Fibrosis

*Background*

Idiopathic pulmonary fibrosis is a devastating disease of unknown aetiology. It has been defined as "a specific form of chronic fibrosing interstitial pneumonia limited to the lung and associated with the histological appearance of usual interstitial pneumonia (UIP) on surgical (thoracoscopic or open) lung biopsy" (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias, 2002). The term IPF was previously applied to all cases of pulmonary fibrosis that did not have a recognised cause. However, as stated in its definition, above, it is currently reserved for, and is synonymous with, the pathological diagnosis of UIP following lung biopsy (in the absence of another known cause of UIP). The prevalence has been classically quoted as 20.2 cases per 100,000 for males and 13.2 cases per 100,000 for females (Coultas *et al*., 1994), however, this appears to be rising and may be as high as 227 per 100,000 among patients aged 75 years and over (Raghu *et al*., 2006). Interestingly and alarmingly the mortality appeared to rise by approximately 5% per year between 1993 and 2002 (Olson *et al*., 2007). The overall prognosis is poor, with a median survival of 3 to 5 years after diagnosis (Khalil *et al*., 2004). Therapies are of limited benefit, if any (King *et al*., 2000; Walter *et al*., 2006).

*Clinical Features*

Accurate diagnosis of IPF is essential in light of the prognostic differences that exist between it and the other IIPs. Clinical diagnosis is currently based on a detailed medical history and physical examination. Most respiratory physicians will be adept at recognising the typical symptoms and signs even before proceeding to further investigation. Further investigation is in the form of a chest X-ray, lung function and high-resolution computed tomography (HRCT) scanning. Clinically, IPF is usually suspected in any patient over 50 years of age presenting with unexplained dyspnoea on exertion that has been present for over 3 months, together with evidence of bibasilar,

inspiratory “velcro” crackles on auscultation of the chest. In addition to at least three of these minor diagnostic criteria, patients must meet all four of the major criteria which include: 1.) exclusion of other known forms of DPLD; 2.) abnormal lung function tests that demonstrate evidence of a restrictive pattern together with reduced gas transfer; 3.) bibasilar reticular opacities on HRCT with minimal ground-glass changes; 4.) bronchoalveolar lavage (BAL) or transbronchial lung biopsy which does not suggest an alternative diagnosis (American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement, 2000). It is important to note that while a normal chest X-ray does not exclude the presence of IPF, radiographic evidence of reduced lung volumes, reticular opacities and honeycombing is extremely suggestive. An alternative diagnosis to IPF is usually suggested when there is radiographic evidence of lymphadenopathy, alveolar opacification or pleural disease.

IPF typically occurs in people aged over 50 years. It usually has an insidious onset characterised, as already discussed, by unexplained dyspnoea on exertion. It is often accompanied by a non-productive cough that develops over a period of about 3 months (Turner-Warwick *et al*., 1980a). In most patients IPF will follow a progressive course which may lead to peripheral oedema and right heart failure. The prognosis is poor, with mortality approaching 70% at 5 years (du Bois *et al*., 2007), which is considerably worse than the other DPLDs. (American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement, 2000).

*Acute Exacerbations of IPF*

The prognosis appears to be significantly worse in patients who develop an exacerbation of IPF, defined as “an acute, clinically significant deterioration of unidentifiable cause in a patient with underlying IPF” (Collard *et al*., 2007). In this group of patients increasing dyspnoea usually occurs within a period of 30 days (Akira *et al*., 1997). Cough, fever and “’flu-like” symptoms may be present (Ambrosini *et al*., 2003; Kim DS *et al*., 2006). Patients often present with severe hypoxia and discussions regarding the need for mechanical ventilation are common. Increased plasma or serum levels of interleukin

(IL)-8 (Ziegenhagen *et al*., 1998), α-defensins (Mukae *et al*., 2002) and soluble ST2 protein (Tajima *et al*., 2003) suggest that activated T-cells and neutrophils may be important during this stage of disease. In keeping with this an increase in BAL fluid (BALF) neutrophils has been reported (Ambrosini *et al*., 2003; Kim DS *et al*., 2006). Analysis of the data from these studies confirm that there is an increase in neutrophils compared with healthy controls, however there is usually also an increase in lymhocytes, and moreover it should be noted that macrophages still remain the most populous cell-type within BAL during exacerbations of IPF. In addition, as will be discussed later, increased proportions of $CD14^{+}CD163^{+}$ circulating blood monocytes have been found in patients with exacerbations of IPF highlighting the potential importance of this cell population (Murray *et al*., 2010). In terms of biomarkers, serum neutrophil elastase, KL-6 and lactate dehydrogenase have been suggested as possible candidates (Yokoyama *et al*., 1998). Radiologically, early reports described diffuse ground-glass abnormalities on chest X-ray (Kondoh *et al*., 1993). HRCT scanning generally demonstrates bilateral ground-glass changes with or without areas of consolidation superimposed on the typical bibasilar sub-pleural reticular changes, traction bronchiectasis and honeycomb abnormalities characteristic of IPF (Akira *et al*., 1997; Ambrosini *et al*., 2003; Kim DS *et al.*, 2006). Three HRCT patterns of abnormality have been suggested: peripheral, multi-focal and diffuse ground-glass (Akira *et al*., 1997). The diffuse and multi-focal patterns have been associated with diffuse alveolar damage (DAD) superimposed on UIP on surgical lung biopsy. Degree of CT involvement may be a predictor of survival (Akira *et al*., 1997; Ambrosini *et al*., 2003; Kim DS *et al*., 2006). Treatment has generally been in the form of high-dose corticosteroids but no randomised controlled trials have been performed. Cyclosporin A has been used, but no convincing evidence of efficacy has come out of those studies (Homma *et al*., 2005; Okamoto *et al*., 2006). If an effect on reducing exacerbations of IPF is analysed in patients undergoing treatment for IPF per se, then anti-coagulant therapy (with warfarin in the out-patient setting or low molecular weight heparin for patients re-hospitalised with severe respiratory failure) or anti-fibrotic with pirfenidone have been shown to have the effect of reducing exacerbation rate; patients treated with anti-coagulant therapy over a 3 year period had a

lower mortality from acute exacerbations than untreated patients (18% versus 71%) (Kubo *et al*., 2005). Patients treated with pirfenidone had no exacerbations compared with untreated patients (Azuma *et al*., 2005). It should be noted that the anti-coagulant trial was not blinded and had a large differential drop-out rate in the treatment arm, and that in the pirfenidone trial small numbers of patients were used, making definite conclusions difficult.

*Therapy*

The lack of an efficacious treatment is one of the reasons why the mortality for IPF is so dreadful. It is my belief that this may reflect a fundamental lack of understanding of not only the history and course of the disease but the pathogenesis. A number of completed and ongoing trials for patients with IPF have been and are being performed and they are summarised in Table 1.1 and Table 1.2, respectively. A brief run-through of some of the trials is now discussed.

As IPF was originally thought to be an inflammatory alveolitis, most of the original trials used "anti-inflammatory" agents. Corticosteroids were one of the first groups of drugs to be used. Many retrospective reviews and case series have been published. In some studies transient objective clinical response was found in a minority of patients, but with no survival benefit relative to untreated patients (Turner-Warwick *et al*., 1980b; Mapel *et al*., 1996; Flaherty *et al*., 2001a). However most of the studies were performed at a time that pre-dated the 2002 classification statement (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias, 2002) and as such many patients who today would not be considered to have IPF would likely have been included in the studies. The data is therefore of dubious quality and a recent systematic review found no high-quality prospective trials on which to base any recommendations (Richeldi *et al*., 2003).

| Company | Comparison | Mechanism of action | Study design | Outcome | Comment | Refs |
|---|---|---|---|---|---|---|
| Investigator | Prednisolone versus prednisolone plus cyclophosphamide | Anti-inflammatory | Randomized, open-label | No difference | Included connective-tissue disease | 74 |
| Investigator | Prednisone plus azathioprine versus prednisone plus placebo | Anti-inflammatory | Double-blind, placebo-controlled | Better lung function change in the azathioprine group | Small number of patients; no significant differences, only trends | 33 |
| Investigator | Prednisone versus colchicine | Anti-inflammatory and antifibrotic | Randomized, open-label | No difference | None | 34 |
| Investigator/ Boehringer–Ingelheim | Prednisolone plus IFNγ versus prednisolone alone | Anti-inflammatory, antifibrotic and pro-$T_H1$ | Open-label, randomized | Improvement in lung function indices in the IFNγ group | Small number of patients; surprising extent of improvement for an IPF group | 35 |
| InterMune | IFNγ versus placebo | Anti-inflammatory, antifibrotic and pro-$T_H1$ | Double-blind, placebo-controlled, randomized | No difference | Low dose corticosteroids allowed | 38 |
| InterMune | IFNγ versus placebo | Anti-inflammatory, antifibrotic and pro-$T_H1$ | Double-blind, placebo-controlled, randomized | No difference | None | 39 |
| Investigator | Prednisolone plus anticoagulation versus prednisolone alone | Anti-inflammatory and anticoagulant | Open-label, randomized | Significant increase in survival in the anticoagulant group | Concerns include disease definition and lack of randomization | 48 |
| Zambon | Azathioprine plus prednisone plus *N*-acetylcysteine versus azathioprine plus prednisolone plus placebo | Anti-inflammatory, antifibrotic and antioxidant | Randomized, double-blind, placebo-controlled | Reduced forced vital capacity and gas transfer for carbon monoxide deterioration in the *N*-acetylcysteine group | Criticized for absence of a total placebo group | 43 |
| Investigator | *N*-acetylcysteine versus bromhexine | Antioxidant and antifibrotic | Open-label, randomized | *N*-acetylcysteine group showed greater oxygen saturation, CT changes and serum KL6 (a mucin gene glycoprotein) | Inhaled route; unusual indices of change | 75 |
| Shionogi | Pirfenidone | Anti-inflammatory, antioxidant and antifibrotic | Double-blind, randomized, placebo-controlled | Positive effect in the pirfenidone group on lung function variables (secondary end points) | Unusual primary end points, which were not met 22% dropped out of the study | 42 |
| Boehringer–Ingelheim | Prednisolone plus IFNγ versus prednisolone plus colchicine | Anti-inflammatory, antifibrotic and pro-$T_H1$ versus anti-inflammatory and antifibrotic | Open-label, randomized | Better survival and lung function in the IFNγ group | Small study | 37 |
| Wyeth | Etanercept (recombinant soluble TNF receptor) | Blocks TNF | Double-blind, randomized, prospective, placebo-controlled | No difference | Exploratory combined pulmonary function variable showed a trend to benefit for etanercept | 47 |
| Actelion | Bosentan | Dual endothelin receptor anatagonist | Double-blind, randomized, placebo-controlled | No difference | Possible benefit for a subset of subjects in the bosentan group | 40 |

**Table 1.1. Completed randomised studies of treatment for IPF**

From: du Bois RM. Strategies for treating idiopathic pulmonary fibrosis. *Nat Rev Drug Discovery*. 2010;9(2):129-40.

| Company | Study agent* | Mechanisms of action | Subject numbers (Phase) | Study design | Goal | Status |
|---|---|---|---|---|---|---|
| Actelion | Bosentan | Dual endothelin receptor antagonist | 600 (III) | Randomized, double-blind, placebo-controlled | To show that bosentan delays disease worsening or death | Active, not recruiting |
| Shionogi | Pirfenidone | Anti-inflammatory, antioxidant and antifibrotic | 250 (III) | Randomized, double-blind, placebo-controlled | To show that pirfenidone reduces the rate of lung function decline | Completed and published[76] |
| Novartis | QAX576 | Blocks IL-13 | 50 (II) | Open-label | To investigate how QAX576 affects levels of IL-13 | Recruiting |
| FibroGen | FG-3019 | CTGF-specific antibody | 27 (I) | Open-label | To determine the safety, pharmacokinetics and biological activity of FG-3019 | Completed |
| Pfizer | Sildenafil | Pulmonary vasodilator | 50 (IV) | Randomized, double-blind, placebo-controlled | To determine the benefit of sildenafil in the peri-lung transplantation setting in IPF cohorts with resting or exercise PAH | Recruiting |
| Novartis | Imatinib | Tyrosine kinase inhibitor | 120 (II–III) | Randomized, double-blind, placebo-controlled | To evaluate the safety and efficacy of imatinib | Completed |
| InterMune | Pirfenidone | Antifibrotic, antioxidant and anti-inflammatory | 779‡ (III) | Randomized, double-blind, placebo-controlled | To assess the efficacy and safety of pirfenidone | Completed; reported at an international meeting |
| Pfizer/NHLBI | Sildenafil | Pulmonary vasodilator | 170 (III) | Randomized, double-blind, placebo-controlled | To evaluate the effectiveness of sildenafil | Active, not recruiting |
| Actelion | Iloprost | Pulmonary vasodilator | 50 (II) | Randomized, double-blind, placebo-controlled | To determine the safety of iloprost inhalation solution in subjects with IPF and PAH | Completed |
| Centocor | CNTO-888 | A human CCL2-specific monoclonal antibody | 120 (II) | Randomized, double-blind, placebo-controlled | To determine the safety and effects of CNTO-888 | Recruiting |
| Lung Rx | Treprostinil | Pulmonary vasodilator | 16 (II) | Open-label | To establish the single-dose tolerability of inhaled treprostinil in IPF patients with PAH | Recruiting |
| Gilead | Ambrisentan | Endothelin A receptor antagonist | 600 (III) | Randomized, double-blind, placebo-controlled | To evaluate the safety and efficacy of ambrisentan in early IPF | Recruiting |
| Boehringer Ingelheim | BIBF-1120 | Triple growth factor kinase inhibitor | 400 (II) | Randomized, double-blind, placebo-controlled | To evaluate the safety and effectiveness of BIBF-1120 in IPF | Active; recruitment complete |
| NHLBI | Prednisone, azathioprine, and *N*-acetylcysteine | Anti-inflammatory, antioxidant and anti-fibroproliferative | 390 (III) | Randomized, double-blind, placebo-controlled | To evaluate the effectiveness of the combination of agents | Recruiting |
| NHLBI | Warfarin | Anticoagulation | 256 (III) | Randomized, double-blind, placebo-controlled | To evaluate the effect of anticoagulation on mortality, hospitalization or decline in FVC of >10% | Not yet open |

**Table 1.2. Ongoing and planned clinical trials for IPF**

From: du Bois RM. Strategies for treating idiopathic pulmonary fibrosis. *Nat Rev Drug Discovery*. 2010;9(2):129-40.

Azathioprine is a purine analogue, converted to mercaptopurine in body tissues, which inhibits adenine deaminase. It is a typical steroid-sparing anti-inflammatory agent. A Cochrane review (Davies *et al*., 2003) identified two case-controlled or randomised controlled trials that evaluated azathioprine (Winterbauer *et al.*, 1978; Raghu *et al*., 1991). Raghu and colleagues compared 13 patients treated with high-dose prednisolone and placebo versus 14 patients treated with an identical prednisolone regimen and azathioprine. These cases were not explicitly identified as having IPF as currently defined; however, the inclusion criteria were similar to what is used today (American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement, 2000). At 1 year, there was no significant difference in clinical measures or mortality. In a secondary analysis adjusted for age, the authors found that there was a marginally significant mortality benefit with azathioprine. Of two lesser quality, earlier randomised controlled trials performed in a heterogeneous population, the study by Winterbauer, mentioned above, showed a marginal benefit of azathioprine. They performed a prospective open label uncontrolled trial that followed 20 patients with variable forms of IIP that were treated initially with prednisolone followed by azathioprine. Twelve out of the 20 patients had improved their vital capacity (VC) by more than 20%, and in the 16 who survived for more than a year there were statistical improvements in forced vital capacity (FVC), resting and exercise $PaO_2$. However, this was a heterogeneous cohort of patients and it was an uncontrolled, open label trial. The results must therefore be interpreted with caution. The study by Fulmer and colleagues (Fulmer *et al*., 1978) was only ever reported in abstract form; they evaluated the effects of addition of azathioprine to prednisolone in a randomised, double-blinded, placebo controlled fashion. They concluded that there were no differences in outcome and considered azathioprine of no benefit, however there was limited data provided in the abstract and again interpretation of results must be with caution. A limitation of the data on azathioprine is the lack of a no-treatment arm. When the decline in FVC in the European Idiopathic Pulmonary Fibrosis International Group Exploring N-acetyl cysteine I Annual (IFIGENIA) trial (prednisolone plus azathioprine) is compared to the decline in FVC in the placebo arm of the GIPF-001 trial (Raghu *et al*., 2004), there is no

obvious difference. This suggests that prednisolone plus azathioprine may be no better than placebo. However, comparison between studies has inherent problems, and examination of FVC is but one outcome measure. However, it is an important point to highlight and raises many potential questions.

Cyclophosphamide is a cytotoxic agent that has been studied. The Cochrane review referred to earlier (Davies *et al*., 2003) only included one study assessing the effects of cyclophosphamide as worthy of further review. This study by Johnson and colleagues (Johnson *et al*., 1989), which was only provisionally included, compared prednisolone alone with a combination of cyclophosphamide plus low-dose prednisolone in a randomised study. In total 43 patients with previously untreated "fibrosing alveolitis" were included. No distinction was made between patients with UIP/IPF and those with other forms of IIP, of which at least 20% were associated with connective tissue disease. Among this heterogeneous group, no differences in clinical markers or mortality rates were seen, although in a secondary analysis, when time to death or "failure of first treatment regimen" was analysed as a single variable, a significant mortality benefit emerged.

A number of other novel therapies have also been assessed for effectiveness in treating IPF. The IFIGENIA trial alluded to earlier compared acetylcysteine, an anti-oxidant and cellular detoxifying agent, with azathioprine and high-dose prednisolone, to azathioprine plus prednisolone. The rate of decline in FVC (9% relative reduction) and DLco (24% relative reduction) at 12 months was significantly reduced in the groups receiving acetylcysteine. No mortality benefit was seen. These effects, whilst encouraging, are of uncertain clinical significance. Furthermore, as acetylcysteine was added to prednisolone and azathioprine, it is difficult to know whether the effects were due to acetylcysteine alone, the combination, or in fact whether prednisolone and azathioprine may have masked acetylcysteine's true effects. The reasons for this assertion will become clear later in relation to the potential effects of corticosteroids on macrophage activation state.

There have now been two large double-blind, placebo-controlled trials assessing the effects of interferon-γ-1b on IPF (Raghu *et al*., 2004; King *et al*., 2009). The GIPF-001 trial (Raghu *et al*., 2004) assessed the effects of interferon-γ-1b on progression-free survival, defined as the time-to-disease-progression, or death, as well as pulmonary function and quality-of-life (QoL) in 330 patients with IPF. Over a median of 58 weeks there was a trend towards improvement in the primary-end point of improved survival in the interferon group, however among patients with less severe lung impairment at baseline there was a significant survival benefit in the interferon group (4% mortality in the interferon group versus 12% in the placebo group). There were however no treatment beneficial effects on lung function, gas exchange, or QoL. As a result of these promising effects, the second interferon-γ-1b trial, the (effect of) interferon-γ-1b on survival in patients with idiopathic pulmonary fibrosis (INSPIRE) trial was designed. However, this study was stopped prematurely following a planned interim analysis; the results showed that after a median duration of 64 weeks on treatment, 80 (of 826 randomised) (15%) patients on interferon-γ-1b and 35 (13%) on placebo had died. The authors concluded that treatment with interferon-γ-1b cannot be recommended as it refuted the sub-group analyses of survival from the GIPF-001 trial.

Etanercept is a soluble tumour necrosis factor (TNF) receptor antagonist that has been used to effectively treat rheumatoid arthritis (Moreland *et al*., 1999). The trial of etanercept was an international randomised, double-blind, placebo-controlled study in which 88 patients received etanercept or placebo, and were assessed after 48 weeks for change in FVC, DLco, and alveolar-arterial oxygen gradient (Raghu *et al*., 2008). No changes in the efficacy end-points were observed between the groups. A non-significant reduction in disease progression was seen in several physiologic, functional and QoL end-points among patients who received etanercept. The authors concluded that the decreased rate of disease progression observed in these measures warrants further evaluation.

Colchicine has been shown to block alveolar macrophage release of fibronectin and insulin-like growth factor-1 (IGF-1) (Rennard *et al*., 1988). It has been utilised in a number of clinical studies in patients with IPF, but none have shown a significant difference in the rate of decline of lung function or improvement in survival when patients were treated with colchicine, with or without corticosteroids (Peters *et al*., 1993; Addrizzo-Harris *et al*., 1997; Douglas *et al*., 1997; Selman *et al*., 1998; Douglas *et al*., 1998; Douglas *et al*., 2000). The best prospective controlled clinical trial (Douglas *et al*., 1998) demonstrated that colchicine was no more effective than prednisolone when the outcomes of survival and pulmonary function were analysed.

Two novel therapies have recently shown some promise. Bosentan is a dual endothelin receptor antagonist (Clozel *et al*., 1994). In the randomised, double-blind, placebo-controlled bosentan use in interstitial lung disease (BUILD-1) trial 158 patients were enrolled and received either bosentan or placebo for 12 months (King *et al*., 2008). There was no change in the primary efficacy end-point of 6 minute-walk test between the 2 groups. However, a *post-hoc* analysis of the data showed that in the patients who had a surgical lung biopsy because of atypical HRCT findings and who exhibited very limited honeycomb changes, the difference between the 2 groups did reach statistical difference. In addition there was a trend to a reduction in the rate of decline in the dyspnoea scores of baseline dyspnoea index and transitional dyspnoea index, as well as a trend to reduction in the heath-related QoL index of Medical Outcomes Study Short-Form 36-instrument and respiratory-specific QoL St. George's Respiratory Questionnaire in the sub-group of patients who required a biopsy. In the light of these results the BUILD-3 trial has just finished enrolling. This trial will look at the effects of bosentan versus placebo in patients with less than 5% honeycombing in at least four of six pre-defined lung regions on CT scanning and who required a surgical biopsy to confirm the diagnosis.

Pirfenidone is a pyridone compound (Margolin & Lefkowitz 1994) that has come through the entire bench-to-bedside process, having been previously investigated in

animal models (discussed in more detail in the later section: Models of Pulmonary Fibrosis: *Bleomycin*). The first report of its possible efficacy in humans was from a Phase II study in 1999 in patients with relatively advanced IPF in whom pirfenidone appeared to slow decline in lung function and allowed for a reduction and eventual discontinuation of corticosteroids in most patients (Raghu *et al*., 1999). It has already been discussed that in the study by Azuma and colleagues (Azuma *et al*., 2005) patients treated with pirfenidone did not have an exacerbation of their IPF. It was also shown in that study that in a pre-specified subset of patients who maintained an $SpO_2$ of greater than 80% during a 6 minute walk test at baseline, the lowest $SpO_2$ improved during a repeat test in the pirfenidone group at 6 and 9 months. Moreover, the rate of decline in VC at 9 months was significantly lower in the pirfenidone group. More recently, the study by Taniguchi and colleagues (Taniguchi *et al*., 2010) demonstrated a reduction in the rate of decline in VC in the pirfenidone group. In addition progression-free survival also showed a significant difference between the groups, favouring pirfenidone. The results are compelling; the data from the 2 studies suggests that pirfenidone may slow the progression of IPF. Furthermore, two multi-centre, randomised, double-blind, placebo-controlled studies (CAPACITY 1 and CAPACITY 2) have just been completed and presented at an international meeting. As demonstrated in the study by Taniguchi and colleagues, the CAPACITY 2 trial confirmed a reduction in the rate of decline of FVC in patients treated with pirfenidone after a 72 week treatment period. There was a trend to reduction in the CAPACITY 1 trial. These trials are encouraging, and it will be interesting to see the full results when these trials come to publication.

In summary therefore, a plethora of studies and clinical trials have been performed in patients with IPF. Treatment with broadly acting anti-inflammatory type drugs has really shown no benefit, nor has treatment with drugs that have targeted particular inflammatory- or fibrosis-related molecules. Promising results have been shown for bosentan whose effects may manifest on the pulmonary vasculature. It is interesting to appreciate that pirfenidone has appeared as an agent with some promise. As will be discussed in more detail later pirfenidone's mode of action is unclear, but it leads to a

reduction in expression of pro-collagen I and pro-collagen III (Iyer *et al*., 1999a), decreases TGFβ expression in lung and in BALF (Iyer *et al*., 1999b) and likely has effects on alveolar macrophage driven secretion of platelet derived growth factor (PDGF) (Gurujeyalakshmi *et al*., 1999). It is therefore possibly surprising that colchicine has not had any promising effect as its mode of action is to reduce the production of fibronectin and IGF-1 from alveolar macrophages (Rennard *et al*., 1988). However it should be pointed out that studies with colchicine were performed prior to 2002 and therefore pre-date the change in classification of the IIPs.

It is very possible that the lack of effect of drug therapy reflects our poor understanding of the natural history and pathogenesis of this increasingly common disease. As we better understand its process and realise the potential of cell therapy, we may soon be in a position to better target, modulate and hopefully alter the course of this devastating disease.

*Biology*

The hallmark of UIP/IPF is temporal heterogeneity (that is, areas of established fibrosis interspersed with areas of relatively normal lung, and gradations between these two extremes), architectural loss and chronic scarring accompanied by microscopic honeycomb-like structural change in the sub-visceral pleural region. The fibrosis is present in the interstitial space (the space between the endothelium and the basement membrane, beneath the epithelium), which includes the alveolar walls. Other distinguishing features of UIP/IPF include the relative paucity of inflammation, a hyperplastic epithelium, and the presence of focal collections of fibroblasts, referred to as fibroblastic foci. This has led some investigators to hypothesise that IPF is a disease characterised by repetitive epithelial injury and abnormal repair (Selman *et al*., 2001). The absence of marked inflammatory infiltrates has led to substantial controversy as to the role of inflammation in IPF. This absence of inflammation does not, however, exclude a role for inflammation in the initiation of the injury that subsequently leads to fibrosis (Gauldie 2002; Strieter 2002).

Furthermore, the origin and importance of fibroblastic foci is controversial. Some investigators believe that fibroblastic foci are the areas where active early aberrant wound repair and matrix deposition occur (Katzenstein 1998), and they have been shown to be independent predictors of lung function and mortality in IPF (King *et al*., 2001). It has recently been suggested that they are not discrete foci but in fact represent an organised reticulum that courses through the lung (Cool *et al*., 2006). Interestingly, this reticulum is surrounded by an extensive capillary network, which suggests that vascular remodeling is an important component of pulmonary fibrosis (Cool *et al*., 2006).

Within these foci are fibroblasts and myofibroblasts. Whatever the initial insult that results in the generation of these foci, many investigators believe that it is predominantly these cells that are responsible for the deposition of matrix and remodelling that is seen in the lungs. These cells are believed to arise from at least three potential sources (Figure 1.2): Trans-differentiation of resident fibroblasts into myofibroblasts (Zhang *et al*., 1994), recruitment of blood-borne fibrocytes (Abe *et al*., 2001; Phillips *et al*., 2004) or bone-marrow derived progenitor cells (Hashimoto *et al*., 2004), or trans-differentiation of resident epithelial or endothelial cells to fibroblasts/myofibroblasts, so called epithelial-mesenchymal transition (EMT) (Willis *et al*., 2005; Kim *et al*., 2006) and endothelial-mesenchymal transition (Hashimoto *et al*., 2009), respectively. This will be discussed in more detail later in this section. Interestingly, fibrocytes may be an independent predictor of early mortality in IPF (Moeller *et al*., 2009).

The predominant hypothesis regarding the pathogenesis of pulmonary fibrosis centres around the epithelial injury hypothesis in which fibrosis is believed to result from epithelial injury, activation and/or apoptosis, with subsequent abnormal wound healing (in the absence of chronic inflammation) (Selman *et al*., 2001; Selman *et al*., 2004). This hypothesis suggests that recurrent unknown injuries to distal lung causes repeated epithelial injury and apoptosis. There is a consequential loss of alveolar epithelial cells which exposes the basement membrane to oxidative damage and degradation. Evidence

for this theory has been established using the bleomycin model of pulmonary fibrosis which results in alveolar epithelial cell injury, apoptosis and necrosis (Hagimoto *et al*., 1997; Li *et al*., 2003). Inhibiting apoptosis reduces collagen deposition and fibrosis (Wang *et al*., 2000). Furthermore in response to bleomycin-induced injury, fibrosis is reduced in the absence of the pro-apoptotic protein Bcl-2 homology (BH3) interacting domain death agonist (BID) despite similar levels of inflammation, injury and active transforming growth factor-$\beta_1$ (TGF$\beta_1$) in BALF (Budinger *et a*l., 2006). Moreover, there is immunohistochemical evidence of increased expression of pro-apoptotic proteins in the alveolar epithelium of patients with IPF (Plataki *et al*., 2005).

The precise process by which alveolar epithelial cells become activated and communicate with fibroblasts to induce collagen deposition and fibrosis is poorly understood. However, various explanations have been suggested. The first involves the production of fibroblast chemotactic factors by activated alveolar epithelial cells and the release of growth factors involved in re-epithelialisation. Candidate growth factors include TGF$\beta_1$, PDGF, IGF-1 and endothelin. Alveolar epithelial cells may regulate the activity of TGF$\beta_1$ via the effects of the $\alpha_v\beta_6$ integrin; Munger and colleagues demonstrated that the integrin $\alpha_v\beta_6$ binds to the TGF$\beta$ latency associated peptide with resultant activation of TGF$\beta$ (Munger *et al*., 1999). In addition they showed that $\beta_6$ null mice display exaggerated inflammation but are protected from pulmonary fibrosis in response to bleomycin. This suggests that when the alveolar epithelium becomes injured and the basement membrane loses its integrity, it will attempt to re-epithelialise with release of growth factors that may recruit fibroblasts or myofibroblasts. If there is no way of regulating the process, then myofibroblast proliferation and extra-cellular matrix deposition may continue unabated.

Another potential explanation is that the epithelium trans-differentiates into fibroblasts/myofibroblasts, effectively bypassing the need to release fibroblast mitogens, so called epithelial-mesenchymal transition or EMT (Figure 1.2).

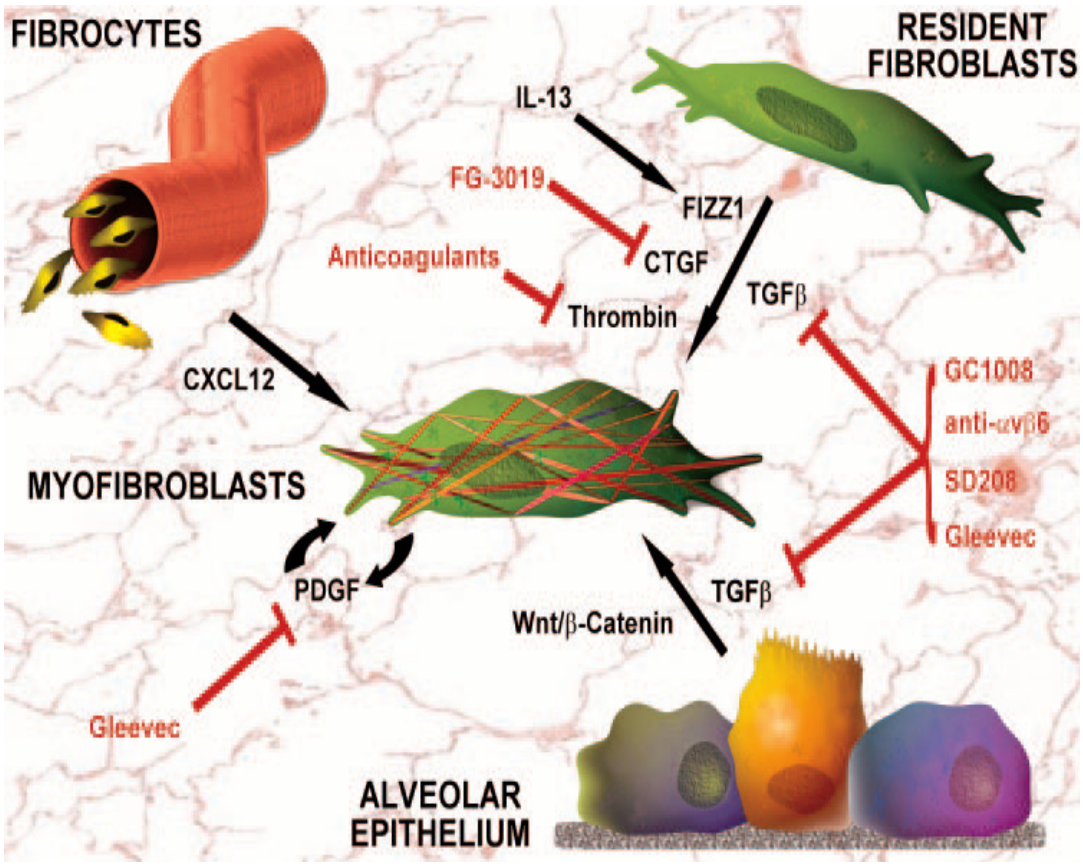



**Figure 1.2. Origins of myofibroblasts in pulmonary fibrosis.**

From: Scotton CJ & Chambers RC. Molecular Targets in Pulmonary Fibrosis: The Myofibroblast in Focus. *Chest* 2007;132:1311-21.

Abbreviations: IL-13=interleukin-13; FIZZ1=Found in inflammatory zone 1 (RELM-α, resistin-like molecule-α); TGFβ=transforming growth factor-β; CTGF=connective tissue growth factor; PDGF=platelet-derived growth factor; CXCL12=chemokine (C-X-C motif) ligand 12; GC1008=human anti-TGFβ monoclonal antibody (MAb); FG-3019=anti-CTGF MAb; SD208=TGFβ receptor 1 kinase inhibitor; gleevec=imatinib, a tyrosine kinase inhibitor.

Interestingly the main inducers of EMT can also promote apoptosis, suggesting that EMT could potentially serve as a pathway for escape from cell death depending on the particular cytokine milieu (Anderson *et al*., 1994; Nicolás *et al*., 2003). EMT is supported by data from several studies. Willis and colleagues analysed the effects of chronic exposure to TGF$\beta_1$ on the phenotype of isolated rat alveolar epithelial cells. In addition, tissue samples from patients with IPF were evaluated for cells co-expressing epithelial (thyroid transcription factor (TTF)-1 and pro-surfactant protein-B (pro-SP-B)) and mesenchymal ($\alpha$-smooth muscle actin ($\alpha$-SMA)) markers. Cells exposed to TGF$\beta_1$ for 6 days demonstrated increased expression of mesenchymal cell markers with a fibroblast-like morphology. In addition TGF$\beta_1$ resulted in increased expression of $\alpha$-SMA, type I collagen, vimentin, and desmin, with associated transition to a fibroblast-like morphology and decreased expression of TTF-1. Cells co-expressing epithelial and mesenchymal markers were abundant in lung tissue from patients with IPF. These results suggested that alveolar epithelial cells undergo EMT when chronically exposed to TGF$\beta_1$, raising the possibility that epithelial cells may serve as a novel source of myofibroblasts in IPF. These results were somewhat corroborated and taken further by the study by Kim and colleagues (Kim KK *et al*., 2006). They generated mice expressing $\beta$-galactosidase exclusively in lung epithelial cells and followed their fates in the adenoviral transforming growth factor-$\beta$ (AdTGF$\beta$) model of pulmonary fibrosis. $\beta$-galactosidase-positive cells expressing mesenchymal markers accumulated within 3 weeks of lung injury. Furthermore, the increase in vimentin-positive cells within injured lungs was nearly all due to $\beta$-galactosidase-positive cells, implicating epithelial cells as the main source of mesenchymal expansion in this model. *Ex vivo*, primary alveolar epithelial cells cultured on provisional matrix components, fibronectin or fibrin, underwent EMT via integrin-dependent activation of endogenous latent TGF$\beta_1$. In contrast, primary cells cultured on laminin-collagen mixtures did not activate the TGF$\beta_1$ pathway and when exposed to active TGF$\beta_1$ underwent apoptosis rather than EMT. These data provide further evidence for the role of EMT during lung fibrosis and implicate the provisional extracellular matrix as a key regulator of epithelial

transdifferentiation during this process. The same group has since gone on to demonstrate that the $\alpha_3\beta_1$ integrin appears to link β-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis (Kim KK *et al*., 2009).

While EMT may provide an explanation for epithelial-mediated production of fibroblasts/myofibroblasts, it does not complete the story in terms of myofibroblast genesis. Endothelial-mesenchymal transition has been proposed as another mechanism for the generation of myofibroblasts (Arciniegas *et al*., 2005). The study by Hashimoto and colleagues (Hashimoto *et al*., 2009) has suggested that endothelial-mesenchymal transition may occur in pulmonary fibrosis. Using Tie2-Cre/CAG-CAT-LacZ double-transgenic mice, in which LacZ was stably expressed in pan-endothelial cells, pulmonary fibrosis was induced by bleomycin administration. Combined X-gal staining and immunocytochemical staining for type I collagen and α-smooth muscle actin revealed the presence of X-gal positive cells in lung fibroblast cultures from bleomycin-treated mice. These findings are the first to show that lung capillary endothelial cells could give rise to significant numbers of fibroblasts through an endothelial-mesenchymal transition in the bleomycin-induced model of lung fibrosis.

The other major source of myofibroblasts, as briefly mentioned previously, is differentiation from pre-existing peri-vascular and peri-bronchial adventitial fibroblasts as well as from submesothelial regions (Figure 1.2). Zhang and colleagues demonstrated that mature fibrotic foci had expanded from their initial localisation within these areas (Zhang *et al*., 1994). The ability, *per se*, of fibroblasts to differentiate into myofibroblasts has been shown *in vitro*; Oda and colleagues established that myofibroblasts and dermal fibroblasts secret similar types of pro-collagen into culture media and that these were predominantly pro-collagen I and pro-collagen III (Oda *et al*., 1988). As a result they proposed that while a myofibroblast has properties of both fibroblasts and smooth muscle cells, it is likely that they are produced by differentiation of fibroblasts. Desmouliere and colleagues subsequently demonstrated that a population of rat and human fibroblasts expressed α-SMA (Desmoulière *et al*., 1992a). Even after

cloning they never found a population of cells that were negative for α-SMA, confirming that the myofibroblasts were indeed differentiated from fibroblasts, rather than arising as a result of contamination. Furthermore they demonstrated that interferon-γ (IFN-γ) lead to a reduction in α-SMA expression as determined by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The same group confirmed that myofibroblasts can be produced from fibroblasts in further studies where they showed that heparin induces the differentiation of fibroblasts into myofibroblasts (Desmoulière *et al*., 1992b).

Circulating fibrocytes are believed to be the other major source of myofibroblasts during pulmonary fibrosis (Figure 1.2). They were originally identified by Bucala and colleagues (Bucala *et al*., 1994) as cells likely derived from hematopoietic stem cells that were classified as $CD45^+$, $CD34^+$, collagen $I^+$. A potential pathogenic role for fibrocytes in IPF has been postulated from experimental models in which blockade of fibrocyte recruitment is protective following experimental induction of lung fibrosis (Phillips *et al*., 2004; Moore *et al*., 2005). Phillips and colleagues described circulating fibrocytes as $CD45^+$, Col $I^+$, $CXCR4^+$. They demonstrated that fibrocytes migrate to the lung following bleomycin administration to mice, and in response to CXCL12. Furthermore, they established that administration of specific CXCL12 neutralising antibodies reduced pulmonary fibrosis, implicating circulating fibrocytes as contributors to the promotion of pulmonary fibrosis. Moore and colleagues established that $CD45^+$, $CD34^+$, Col $I^+$ fibrocytes express CCR2, that lung fibrocytes migrate in culture in response to CCL2, and that CCR2-null mice have less recruitment of these cells in response to fluorescein isothiocyanate (FITC)-induced pulmonary fibrosis. Moreover, adoptive transfer of bone-marrow from wild-type mice to CCR2-null mice exposed to lethal irradiation, followed by FITC-induced pulmonary fibrosis, demonstrated that the fibrotic phenotype was restored. The potential importance of fibrocytes in IPF in predicting outcome has recently (and previously) been described (Moeller *et al*., 2009). However, evidence that fibrocytes are capable of differentiating into fully activated myofibroblasts, especially in patients with IPF, is still lacking.

More recently, however, classical descriptions of the sources of myofibroblasts have been questioned. Tanjore and colleagues have recently attempted to quantify the relative contribution of EMT and bone-marrow-derived circulating precursors to the production of fibroblasts and myofibroblasts (Tanjore *et al*., 2009). Using cell fate reporter mice that had pulmonary fibrosis induced by bleomycin they established that approximately one-third and one-fifth of fibroblasts had arisen as a result of EMT or were derived from bone-marrow precursors, respectively. However they established that EMT and circulating bone-marrow derived precursors were a minor source of lung myofibroblasts. The role of fibroblasts/myofibroblasts in the promotion of pulmonary fibrosis continues to be elucidated, but it is clear that much work will be required before a clear understanding of the sources, function and interactions of these interesting cells is achieved.

## Models of pulmonary fibrosis

*Bleomycin*

The bleomycin model of pulmonary fibrosis is the best characterised model of lung fibrosis. Bleomycin sulphate is a mixture of glycopeptide antibiotics isolated from a strain of *Streptomyces verticillus*, and is comprised primarily of $A_2$ (approximately 70%) and $B_2$ (approximately 30%) (Kross *et al*., 1982) sub-types. It causes oxidant-mediated DNA damage (Chen *et al*., 2008) leading to pulmonary fibrosis (Adamson & Bowden 1974). Pulmonary toxicity is dose dependent but there is minimal myelosuppression or immunosuppression (Cooper *et al*., 1986).

Bleomycin was first given to animals in experimental form by Robert Fleischman and colleagues (Fleischman *et al*., 1971). They administered bleomycin intravenously to dogs (beagles) every fourth day for 11 or 31 treatments depending on the dose administered. A high and a low dose regimen were employed. Ten dogs were treated. Three became moribund; two treated with the higher dosing schedule became unwell on day 38 and 70, and one treated with the lower dosing schedule became unwell on day 137. All dogs developed microscopic evidence of a focal and confluent interstitial pneumonia with a sub-pleural localisation. Gross pulmonary lesions were only apparent in dogs treated for more than 70 days. Additional histopathological features were interstitial fibrosis, marked hyperplasia and metaplasia of type II pneumocytes, focal hyperplasia of pleural mesothelium and a pleomorphic inflammatory infiltrate. Deeper parenchymal areas had no evidence of lung injury or fibrosis. Adamson and Bowden went on administer bleomycin intra-peritoneally to mice, with the aim of using it as a model of pulmonary fibrosis: “The consistency and rapidity with which pulmonary fibrosis is induced by bleomycin administration to animals suggests that this experimental method provides a suitable model for the investigation of pulmonary fibrosis in man” (Adamson & Bowden 1974). As a dose finding experiment they gave between 0.01mg and 1mg. Mortality was 10% for doses of 0.02mg and 0.04mg, 60% for 0.1mg, 30% for 0.5mg and 100% for 1mg. As a result of the dose finding

experiment they subsequently went on to give thirty mice 0.5mg twice weekly for four weeks. Groups of three mice were culled weekly during the injection period and at two-weekly intervals thereafter up to a total of twelve weeks. The earliest microscopic abnormality was detected at two weeks and involved the endothelium and the pulmonary vasculature. The first alveolar abnormalities were detected at four weeks and included necrosis of type I pneumocytes (with no effect on type II pneumocytes). Intra-alveolar and septal fibrosis were also present at four weeks and became more severe and progressive with increasing time. The changes became extensive by eight to twelve weeks. Bleomycin was subsequently given to pheasants (Bedrossian *et al*., 1977), baboons (McCullough *et al*., 1978), hamsters (Snider GL *et al*., 1978a; Snider GL *et al*., 1978b), rats (Thrall *et al*., 1979), guinea pigs (Upreti *et al*., 1979) and rabbits (Laurent *et al*., 1981).

An advantage of the bleomycin model is that it causes inflammatory and fibrotic reactions within a short period of time, this being more pronounced for the intra-tracheal method of administration. The "first" phase of bleomycin lung injury is that of inflammation and this is followed by the "progressive fibrotic" phase. In a 21 day model in rats this switch from inflammation to fibrosis has been scrutinised in some detail (Chaudhary *et al*., 2006). The authors established that during the inflammatory phase there is up-regulation in gene expression of pro-inflammatory cytokines such as IL-1, TNF-$\alpha$, IFN-$\gamma$ and IL-6. This is followed by up-regulation in gene expression of the markers of fibrosis TGF$\beta$, pro-collagen I and fibronectin. In their 21 day model the switch from inflammation to fibrosis occurs around day 9.

The bleomycin model has contributed significantly to our understanding of aspects of the pathogenesis of pulmonary fibrosis. Notably, it has allowed for better understanding of the role played by TGF$\beta$ during pulmonary fibrosis (Zhao *et al*., 2002). In their paper, Zhao and colleagues established that there was reduced pulmonary fibrosis, following bleomycin administration, in Smad3-null mutant mice as determined by histology and hydroxyproline content (hydroxyproline content being a quantifiable

indicator of the degree of pulmonary fibrosis). Furthermore there was a reduction in gene expression of pro-collagen I and fibronectin in the null mutants compared to wild-type. Smad3 is known to be an intra-cellular mediator of TGFβ signalling (Zhang *et al*., 1996). Other groups have shown using the bleomycin model that inhibition of the activity of TGFβ by antibodies (Giri *et al*., 1993), endogenous antagonists (Giri *et al*., 1997; Kolb *et al*., 2001) or soluble TGFβ receptors (Wang *et al*., 1999) results in a reduction in pulmonary fibrosis. The bleomycin model has also been used to elucidate mechanisms of TGFβ activation *in vivo*; within the extra-cellular matrix TGFβ often exists as part of the large latent complex (LLC), which is composed of TGFβ, its inhibitory molecule the latency associated peptide (LAP), and the latent TGFβ-binding protein. As part of this complex, TGFβ is rendered latent, or inactive (Munger *et al*., 1999). As mentioned earlier, Munger and colleagues demonstrated that the TGFβ-LAP is a ligand for the integrin $\alpha_v\beta_6$, that $\alpha_v\beta_6$ activates TGFβ, and that $\beta_6$ null mice are protected from bleomycin-induced pulmonary fibrosis. Horan and colleagues subsequently showed that bleomycin-induced pulmonary fibrosis is reduced, as measured by hydroxyproline quantification, when a monoclonal antibody against the $\alpha_v\beta_6$ integrin is given during the progressive fibrotic phase of bleomycin injury (Horan *et al*., 2008).

Pirfenidone has "recently" emerged as a potentially promising drug for treatment of IPF (Raghu *et al*., 1999; Taniguchi *et al*., 2010; Collard 2010). Its therapeutic potential has been discussed earlier. The promising effects of pirfenidone were initially identified using the bleomycin model. Work from Shri Giri's group established that feeding pirfenidone to hamsters during bleomycin-induced pulmonary fibrosis lead to a reduction in fibrosis as measured by hydroxyproline quantity (Iyer et al., 1995; Iyer *et al*., 1998) or pulmonary function (Schelegle *et al*., 1997). They demonstrated that its effects were partly due to down-regulation in gene expression of pro-collagen I and pro-collagen III (Iyer *et al*., 1999a), and partly due to effects on TGFβ; hamsters given bleomycin and subsequently fed pirfenidone demonstrated a reduction in lung gene

expression of TGFβ and reduction in TGFβ in the BALF (Iyer *et al*., 1999b). Further work established that pirfenidone inhibited synthesis of the PDGF A-and B- isoforms as measured in BALF of hamsters treated with bleomycin (Gurujeyalakshmi *et al*., 1999). Interestingly, alveolar macrophages secrete more PDGF in patients with IPF compared to normal controls (Martinet *et al*., 1987). More recent work suggests that pirfenidone may also work by inhibition of TGFβ-stimulated production of heat-shock protein 47 (HSP47), a collagen-specific molecular chaperone (Nakayama *et al*., 2008).

It is recognized that there exists strain variability in response to bleomycin (Schrier *et al*., 1983) and that this is likely due to strain-specific differences in expression of the inactivating enzyme bleomycin hydrolase (Sebti *et al*., 1989). Depending on the source, dose given, age and strain of mice, fibrosis may peak as much as 2 months after initial bleomycin instillation (Chua *et al*., 2007; Cabrera *et al*., 2007). Although some studies have suggested the presence of differences between the late chronic stages of animal pulmonary fibrosis and features of the human disease (Borzone *et al*., 2001), the bleomycin model undoubtedly represents an invaluable tool to dissect out the mechanisms of pulmonary fibrosis and with which to test *in vivo* molecular agents involved in the pathogenesis of pulmonary fibrosis (Chua *et al*., 2005; Moeller *et al*., 2008; Moore & Hogaboam 2008). It is well characterised, reproducible and has clinical relevance.

*Adenoviral transforming growth factor-β model of lung fibrosis*

Over-expression of TGFβ by use of an adenovirus construct containing porcine $TGF\beta_1$ (AdTGFβ) has been shown to induce pulmonary fibrosis in mice and rats (Sime *et al*., 1997; Warshamana *et al*., 2002). The recombinant adenovirus vector is deleted in the E1 and E3 regions. E1 is indispensable for viral propagation; E1-deleted adenoviruses are therefore replication incompetent (Graham *et al*., 1977). E1-deleted adenoviruses can be grown in 293 cell lines that have been transformed by the E1 region such that they supply the required replication proteins (Bramson *et al*., 1995). The E3 region is required for viral persistence as a result of interactions with major histocompatiability

complex (MHC) class I proteins (Routes & Cook 1990). It is non-essential for viral growth, and as a result can be removed from E1-deleted adenoviral constructs to allow for the insertion of large inserts (Bett et al., 1994). The TGFβ insert contains a mutation of cysteine to serine at positions 223 and 225, rendering the expressed $TGF\beta_1$ biologically active (Sime *et al*., 1997). Control virus (AdDL70-3) was constructed as previously described (Bett *et al*., 1994); it is deleted in E1 and E3, with no additional insert, and thus is an "empty" vector. In this model transgene expression is not significant after day 14 and as such the fibrogenic process initiated by TGFβ over-expression becomes self-perpetuating, a feature of IPF. Furthermore the histopathological characteristics of this model are extensive and persistent fibrosis, beginning in the peri-vascular and peri-bronchial areas, but quickly becoming widespread throughout the lung interstitium, extending up to the pleural surface (Sime *et al*., 1997). There is less inflammation than in the bleomycin model and some researchers believe that this model may mirror more closely the human form of the disease, IPF, and as a result may warrant further use as a model of lung fibrosis.

*Silica*

Administration of silica to induce experimental lung fibrosis has been used in rats (King *et al*., 1953; Goldstein & Webster 1966), guinea pigs (Schepers *et al*., 1957; Dauber *et al.*, 1980) and mice (Hatch *et al*., 1984). Silica has the advantage that it induces lung fibrosis that has associated fibrotic nodules resemblant of those seen in humans exposed to occupational dusts, and the model is of a more persistent nature than other models of lung fibrosis (Davis *et al*., 1998). However, fibrosis can take 12-16 weeks to develop (Davis *et al*., 1998) and probably needs at least 8 weeks (Barbarin *et al*., 2005). Different strains of mice exhibit variable responses to silica with BALB/c mice being resistant (Davis *et al*., 1998) and if aerisolisation is needed then special equipment is required due to the potential toxicity to humans. Furthermore, in specific relation to my studies, silica is known to be toxic to macrophages (Heppleston 1969; Civil & Heppleston 1979). If I had used silica as my model of lung fibrosis, then the mere act of

producing the model would have confounded any interpretation of the results, and for this reason this model was not used in this thesis.

*Fluorescein isothiocyanate (FITC)*

The FITC model of lung fibrosis was first described by Roberts and colleagues (Roberts *et al*., 1995). FITC induces lung fibrosis that typically develops around 14-28 days (Christensen *et al*., 1999) but may occur over several months (Roberts *et al*., 1995), and does not appear to be self-limiting (Fisher *et al*., 2005); it has clinical relevance (Roberts *et al*., 1995) and there appear to be no strain-resistant mice (Christensen *et al*., 1999). In addition it allows direct visualisation of the areas of the lung where collagen deposition occurs when immunofluorescence imaging for the characteristic green color of FITC is performed. However, the effective dose of FITC required to induce lung fibrosis is particularly LOT dependent (Moore *et al*., 2008), and as such there is much variability and no consistent reproducibility when using this model. Furthermore, from a practical point of view the solution of FITC to be administered has to be made fresh each day and must be vortexed before each instillation. This raises concern as to the consistency of fibrosis induced by each administration; add to this the variability of lung fibrosis that arises due to LOT variability and there appear several reasons to be wary of the consistency and reproducibility of this model. This is compounded by the fact that this model has no direct clinical relevance to human pulmonary fibrosis, and for these reasons this model was not used.

*Irradiation-induced lung fibrosis*

Pulmonary fibrosis may be induced by irradiation of mice either in the form of total body irradiation which takes at least 20 weeks to develop (McDonald *et al*., 1993), or by using lead-shielding techniques that ensure that only the thoracic cavity is exposed to irradiation; in this form of injury fibrosis takes at least 24 weeks to develop (Johnston *et al*., 1998; Rube *et al*., 2000). This model has the advantage that there is some clinical relevance. However, there are obvious draw-backs to the length of time it takes for fibrosis to develop and in using a protocol that irradiates the whole body there will

undoubtedly be confounding effects on other parts of the body, in particular on the bone marrow. Furthermore, there is strain-variability in the response to injury (Franko *et al*., 1996; Sharplin & Franko 1989), C3H/HeJ and CBA/J mice being fibrosis resistant and C57Bl/6 mice being fibrosis-prone. Moreover, in C57Bl/6 mice fibrosis may take up to 33 weeks to develop (Haston & Travis 1997) incurring extremely large costs even before any interventions or analysis are performed, and with all of these considerations in mind I did not use this model in any of my studies.

*Miscellaneous*

Many other models of "lung fibrosis" have been employed which include the Fas ligand-induced model (Matute-Bello *et al*., 2001), haptenic antigens (such as immune induced by a recall cell-mediated immune response against the hapten 2,4,6-trinitrobenzene sulphonic acid (TNBS)) (Zhang-Hoover *et al*., 2000), murine γ-herpes virus 68-induced, vanadium pentoxide-induced (Bonner *et al*., 1998) and many more. However, their mechanism of action is often poorly understood, they are generally not as well characterised as the bleomycin and AdTGFβ models, and they have questionable clinical relevance: for these reasons they were not used.

*Reversibility of lung fibrosis*

Attempts have been made to produce "new" reversible models of pulmonary fibrosis. For example, administration of adenoviral-connective tissue growth factor (AdCTGF) induces a reversible lung injury when given to mice (Bonniaud *et al*., 2003; Bonniaud *et al*., 2004). This model has the advantage that fibrogenesis is indeed transient, however uncertainty has been raised as to whether this is genuine model of lung fibrosis as there is no up-regulation of TIMP1, a characteristic marker of fibrosis activity. The bleomycin model of pulmonary fibrosis, as has already been discussed, is the best characterised model of lung fibrosis. It has been known for some time that this model may be self-limiting (Izbicki *et al*., 2002), and indeed previous studies have demonstrated that fibrosis is starting to reverse after 6 weeks and has resolved by 9

weeks (Lawson *et al*., 2005) making it a good model in which to interrogate the resolution phase of pulmonary fibrosis.

In summary, therefore, there exist many models of pulmonary fibrosis, each of which has advantages and disadvantages. It is clear that some models are more reproducible and of better clinical relevance. In examining all of the differences that exist, I aimed to use models with direct clinical relevance, that are clearly practical and reproducible, and that have been well characterised so as to ensure that any criticism of the results would focus on the results *per se*, rather than any inherent problem with the model itself. For this reason I decided to use the bleomycin and AdTGFβ models to investigate the role of monocytes and macrophages during lung fibrogenesis, and the bleomycin model to investigate the role of macrophages during fibrosis resolution.

**Lung macrophages**

The alveolar macrophage stands as the "guardian" of the alveolar–blood interface, serving as the front line of cellular defence against respiratory pathogens (Sibille & Reynolds 1990). Alveolar macrophages are the primary phagocytes of the innate immune system, clearing the alveoli of infectious, toxic, or allergic particles that have evaded the mechanical defences of the respiratory tract. Alveolar macrophages function as regulators of alveolar defence against respiratory infection (Dockrell *et al*., 2003; Dockrell & Whyte 2006) and have an equally important role in resolving inflammation within the airspace (Haslett 1999; Gilroy *et al*., 2004; Marriott *et al*., 2006).

Alveolar macrophages attempt to keep their environment relatively quiescent by down-regulating immune responses (Holt 1986). It is believed they can subsequently adapt their activity to mount an immune response when required (Lambrecht 2006).

Initial studies suggested that local proliferation of precursors was the main source of alveolar macrophages under normal conditions; studies in radiation bone-marrow transplant chimera mice found that if a radiation protocol that is less toxic to lungs was used (while having the same effect on bone marrow), then most alveolar macrophages were of host origin almost 1 year after treatment (Tarling *et al*., 1987). More recently studies using bone marrow chimeric CD45.2 mice that were generated by lethal irradiation of CD45.2 alloantigen-expressing recipient mice and bone marrow transplantation from CD45.1 alloantigen-expressing donor mice, confirmed that in resting steady state conditions resident alveolar macrophages (of untreated chimeric CD45.2 mice) are very slowly replaced, however in this instance by constitutively immigrating CD45.1 positive monocytes. Replacement occurred at a rate of 40% by 1 year (Maus *et al*., 2006). However, after treatment with *Escherichia coli* endotoxin lipopolysaccharide (LPS) more than 85% of the resident CD45.2 positive alveolar and interstitial macrophages were exchanged by donor CD45.1-expressing macrophages within 2 months (Maus *et al*., 2006). The same group showed using similar techniques that following infection with *Streptococcus pneumoniae*, 80% of alveolar macrophages were replaced by donor cells after 7 days (Taut *et al*., 2008). Another study demonstrated that in the resting steady state after whole-body irradiation and transfer of green fluorescent protein (GFP)-labelled bone marrow, host alveolar macrophages were replaced with cells of donor origin (74.5% of total bronchoalveolar lavage cells were of donor origin by 60 days and 80.4% by 90 days) (Matute-Bello *et al*., 2004). The differences in the studies likely reflect differences in methodology with respect to the quantity and dosing schedule of radiation, the radiation type, and the methods employed to detect cellular turnover. It is likely that alveolar macrophage turnover is stimulus dependent, and it is clear that alveolar macrophages can be replaced by recruited macrophage precursors. A possible mechanism by which this occurs is highlighted by work suggesting that alveolar macrophages originate from blood monocytes and that the process requires a lung-resident intermediate (Landsman & Jung 2007). This idea was originally proposed by Bowden and colleagues (Bowden *et al*., 1969); they suggested that alveolar macrophages originate from a pulmonary interstitial cell precursor

(Bowden *et al*., 1972). Landsman and Jung proposed that the generation of alveolar macrophages involves the differentiation of blood monocytes into macrophages in the lung parenchyma, proliferative expansion of these cells, and their emigration into the alveolar space. In such a scenario, lung interstitial macrophages may serve as a local reservoir from which alveolar macrophages can be generated when needed.

Several studies have attempted to phenotype the different sub-populations of pulmonary macrophage. Steady state or resting alveolar macrophages are believed to be $CD11c^{hi}$MHC-$II^{int}CD11b^{lo}$ (Strauss-Ayali et al., 2007). Depletion of lung $CD11c^{+}$ cells (macrophages and dendritic cells) results in subsequent repopulation of the murine alveolar macrophage population by cells which are derived from the $CX3CR1^{hi}CCR2^{-}$ $Ly6C^{lo}$ ($CD14^{+}CD16^{+}$ in humans) blood monocyte subpopulation (Landsman et al., 2007). However, a population of $CD11c^{hi}CD11b^{hi}$ lung macrophages has also been described (Kirby *et al*., 2006), and as such the distinction between lung macrophages and dendritic cells may not be as straightforward as at first glance.

**Macrophage activation states**

Recent work by many groups has shown the heterogeneity that macrophages exhibit, culminating in an attempt to categorise macrophages into subtypes, or activation states, according to protein and RNA expression patterns, function, and the stimuli required to induce these activation states (Wynn 2004; Gordon & Taylor 2005).

Several activation states have been described:

Classically activated or M1 macrophages develop in response to stimulation with IFN-γ and LPS (Adams 1989). IFN-γ is predominantly secreted by $CD8^{+}$ cytotoxic T-lymphocytes, antigen-presenting cells, natural killer (NK) cells, and to a lesser extent by B cells. LPS is the major component of the outer membrane of Gram negative bacteria.

It binds to the serum protein LPS-binding protein (LBP) and is transferred to the myeloid marker antigen, CD14, at the cell surface. LPS then interacts with toll-like receptor 4 and its accessory protein MD2 (Guha & Mackman 2001) and stimulates the activation of various mitogen-activated protein kinase (MAPK) pathways such as the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways. M1 macrophages display widespread morphology depending on their tissue location, but are typified by the production of pro-inflammatory cytokines such as IL-1β, TNFα and IL-12 (Trinchieri *et al*., 2003). They also secrete the chemokines CCL15, CCL20 (macrophage inhibitory protein-3α (MIP-3α)), CXCL13, and the angiostatic IFN-γ-responsive chemokines CXCL9, CXCL10/IP-10, and CXCL11 that are known to co-ordinate NK and T-helper 1 cell recruitment in type I immune responses through their activity on CXCR3 (Mantovani *et al*., 2004; Strieter *et al*., 2005a; Strieter *et al*., 2005b; Martinez *et al*., 2006). Furthermore, M1 macrophages display elevated levels of expression of MHC class II and co-stimulatory molecules CD80 and CD86, as well as low levels of Fcγ RII (Boehm *et al*., 1997). Functionally, M1 cells are characterised by enhanced endocytosis and an enhanced ability to kill intracellular pathogens (Mosser & Handman 1992). This increased microbicidal activity is mediated by different mechanisms such as restriction of iron and other nutrients, acidification of the phagosome (Carlin *et al*., 1989; Gruenheid & Gros 2000), and release of nitric oxide (NO) from L-arginine by virtue of iNOS activity, a macrophage specific IFN-γ-inducible isoform of NO synthase (MacMicking *et al*., 1997), and a marker of M1 macrophage activation.

Macrophages can also be activated by direct recognition of microbial pathogen-associated molecular patterns, such as LPS, through germ-line encoded pattern recognition receptors (PRR) (Janeway & Medzhitov 2002), such as toll-like receptors (TLRs) and nucleotide oligomerisation domain (NOD) receptors (Mukhopadhyay & Gordon 2004). This activation phenotype has been referred to as *innate* activation (Mukhopadhyay *et al*., 2006) and resembles M1 activation to a certain extent, as it is characterised by secretion of pro-inflammatory cytokines, such as IL-1β and TNFα, and

increased expression of co-stimulatory molecules. However, innate activated macrophages do not show the same level of phagocytosis. They express a different pattern of membrane receptors for pathogens, which includes the macrophage receptor with collagenous structure (MARCO), a novel marker of innate activated macrophages (Mukhopadhyay *et al*., 2006). Innate activation is usually unable to fully develop into an M1 profile because TLR ligation typically induces expression of only low levels of p40 and is therefore insufficient to trigger production of the M1 biologically-active IL-12 (Trinchieri *et al*., 2003). However, complete pathogens can induce functional IL-12 heterodimers, indicating that microbial stimulation can be considered a subgroup within the M1 macrophage group (Skeen *et al*., 1996).

The M2b activation state is produced in culture by ligation of receptors such as the TLRs, most of which are expressed by cells of monocyte–macrophage lineage and are associated with microbicidal activity and pro-inflammatory cytokine production (Gordon and Taylor, 2005). M2b cells are the functional converse of M1 cells, characterised by low IL-12 and high IL-10 production, a cytokine profile which favours the development of type II adaptive immune responses (Anderson & Mosser 2002). M2b macrophages are distinct from M2a macrophages as they produce much higher levels of IL-10, but also produce significant amounts of TNFα, IL-1β, and IL-6, suggesting that these cells are not anti-inflammatory *per se*.

The M2c group includes cells stimulated with IL-10, TGFβ, or glucocorticoids. It is a functionally heterogeneous group of cells that hints at an over-simplification of macrophage activation state classification. M2c macrophages are usually regarded as deactivated macrophages as they are characterised by down-regulation of pro-inflammatory cytokines and increased debris scavenging activity. IL-10 is produced by macrophages, T cells, B cells, and a variety of other cell types including mast cells and keratinocytes, as part of the homeostatic response to infection and inflammation. It plays a critical role in limiting the duration and intensity of immune and inflammatory responses. In macrophages, IL-10 inhibits production of pro-inflammatory cytokines

such as TNFα, IL-6, IL-12 and antigen presentation by monocytes or macrophages via down-regulation of MHC class II and co-stimulatory molecules (de Waal Malefyt *et al*., 1991). TGFβ is a pleiotropic cytokine which mediates a wide variety of effects on cellular differentiation, activation, and proliferation. On macrophages and monocytes TGFβ regulates activation, cytokine production, host defence, and chemotaxis. It inhibits LPS-induced macrophage production of the pro-inflammatory cytokines TNFα, IL-lα and IL-18 (Bogdan *et al*., 1992). Glucocorticoids are released in response to a variety of stressors such as infection and trauma and are essential for the maintenance of homeostatic functions (Valledor *et al*., 2004). They are recognised by the glucocorticoid receptor, which strongly suppresses pro-inflammatory cytokines such as TNFα, IL-1, IL-6, IL-8, IL-12, and pro-inflammatory mediators such as iNOS and cyclo-oxygenase 2 (Valledor *et al*., 2004). Glucocorticoids also inhibit the inflammatory response by increasing the expression of IL-10 (Ehrchen *et al*., 2007) and other molecules with anti-inflammatory functions such as the scavenger receptor CD163 (Högger *et al*., 1998), and by interfering with the IL-1 system as a result of increasing expression of the decoy receptor IL-1RII (Ehrchen *et al*., 2007). Glucocorticoids also down-regulate a number of genes known to be up-regulated by IFN-γ, such as the chemokines CXCL10, CXCL11, CCL5 and the chemokine receptor CX3CR1 (Ehrchen *et al*., 2007).

Of particular interest to fibrotic conditions are the alternatively activated macrophages (M2a) which are induced following stimulation with IL-4 or IL-13. IL-4 and IL-13 appear to be predominantly produced by Th2 cells, mast cells, and basophils (Stein *et al*., 1992). IL-4 binds two receptor complexes: the high-affinity receptor complex composed of the IL-4 receptor-α (IL-4Rα) chain and the IL-2 receptor common γ-chain (Martinez *et al*., 2009), both of which are expressed to varying degrees by macrophages, T cells, B cells and mast cells; and the low-affinity receptor complex composed of the IL-4Rα/IL-13Rα1 heterodimer (Martinez *et al*., 2009) and which is expressed on monocytes, fibroblasts, B cells and endothelial cells. IL-13 binds to three receptor complexes: the low-affinity IL-13Rα1 monomer, the high-affinity IL-13Rα2 monomer,

and the IL-13Rα/IL-4Rα heterodimer (Mueller *et al*., 2002). The biological actions of IL-4 and IL-13 on monocytes and macrophages were for a long time considered to be purely anti-inflammatory, based on their down-regulation of pro-inflammatory mediators such as IL-1β and TNFα (D'Andrea *et al*., 1995), IFN-γ (Mosmann *et al*., 1989), IL-8 (Standiford *et al*., 1990) and superoxide anion production (Abramson *et al*., 1990). In addition, IL-4 decreases the expression of important membrane molecules including CD14 (Lauener *et al*., 1990) and CCR5 (Wang *et al*., 1998). In recent years a number of studies have shown that IL-4/IL-13 also regulate key molecules such as MHC class II (Cao *et al*., 1989; Morga & Heuschling 1996), $\beta_2$ integrins (McNally *et al*., 2007), the chemokines CCL22 (macrophage-derived chemokines (MDC)) (Bonecchi *et al*., 1998) and CCL18/AMAC-1 (Kodelja *et al*., 1998), tissue-type plasminogen activator (Hart *et al*., 1989a) and matrix metalloproteinase 1 (Chizzolini *et al*., 2000). In a recent genome-wide M1/M2a transcriptional comparison study it was demonstrated that IL-4 induces up-regulation of several scavenger receptors and C-type membrane lectins, including MRC1, SR-A, Dectin-1, DC-SIGN, DCIR (CLECSF6), DCL-1, and CLECSF13 (Martinez *et al*., 2006). Alternatively activated macrophages also express fibronectin 1 and matrix associated protein betaIG-H3 (Gratchev *et al*., 2001), which promote fibrogenesis, the coagulation factor XIII (Töröcsik *et a*l., 2005) and IGF-1 (Kodelja *et al*., 1997), which provide signals for tissue repair and proliferation. IL-4/IL-13 also affect the IL-1β system by enhancing the production of IL-1 receptor antagonist and the decoy IL-1β type II receptor (Hart *et al*., 1989b) which synergistically interfere with IL-1β biological activity (Mantovani *et al*., 2001). From murine studies it is recognised that, in contrast to M1 macrophages, M2a macrophages do not express iNOS, but express high levels of arginase 1 (Arg1), which skews the metabolic pathway of NO to the production of proline (Gordon 2003). Consequently, these cells fail to produce NO and are significantly compromised in their microbicidal ability for intracellular pathogens (Modolell *et al*., 1995; Hesse *et al*., 2001), but they synthesise polyamine and proline that stimulate cell growth, collagen formation, and tissue repair and fibrosis (Hesse *et al*., 2001; Gordon 2003). In human *in vitro* activated macrophages this

metabolic signature is absent (Scotton *et al*., 2005). M2a macrophages express high levels of the chemokines CCL13/MCP-4, CCL8/MCP-2, and CCL26/eotaxin-3, which coordinate the recruitment of eosinophils, basophils and some polarised Th2 cells, through the activity on CCR3 (Mantovani *et al*., 2004) and are involved in pro-angiogenic networks (Strieter *et al*., 2005a; Rosenkilde *et al*., 2004). *In vitro* alternatively activated macrophages also express the extracellular matrix-remodelling enzyme matrix metalloproteinase (MMP)-12, which has been demonstrated to be selectively expressed in the late, but not early stages of tuberculosis (Kahnert *et al*., 2006).

It is likely that there is a large degree of overlap within the various groups of macrophage activation, and it is possible that M1 and M2 macrophages represent the activation state of macrophages across a spectrum of activation states (Mantovani *et al*., 2001). This is represented in Figure 1.3.

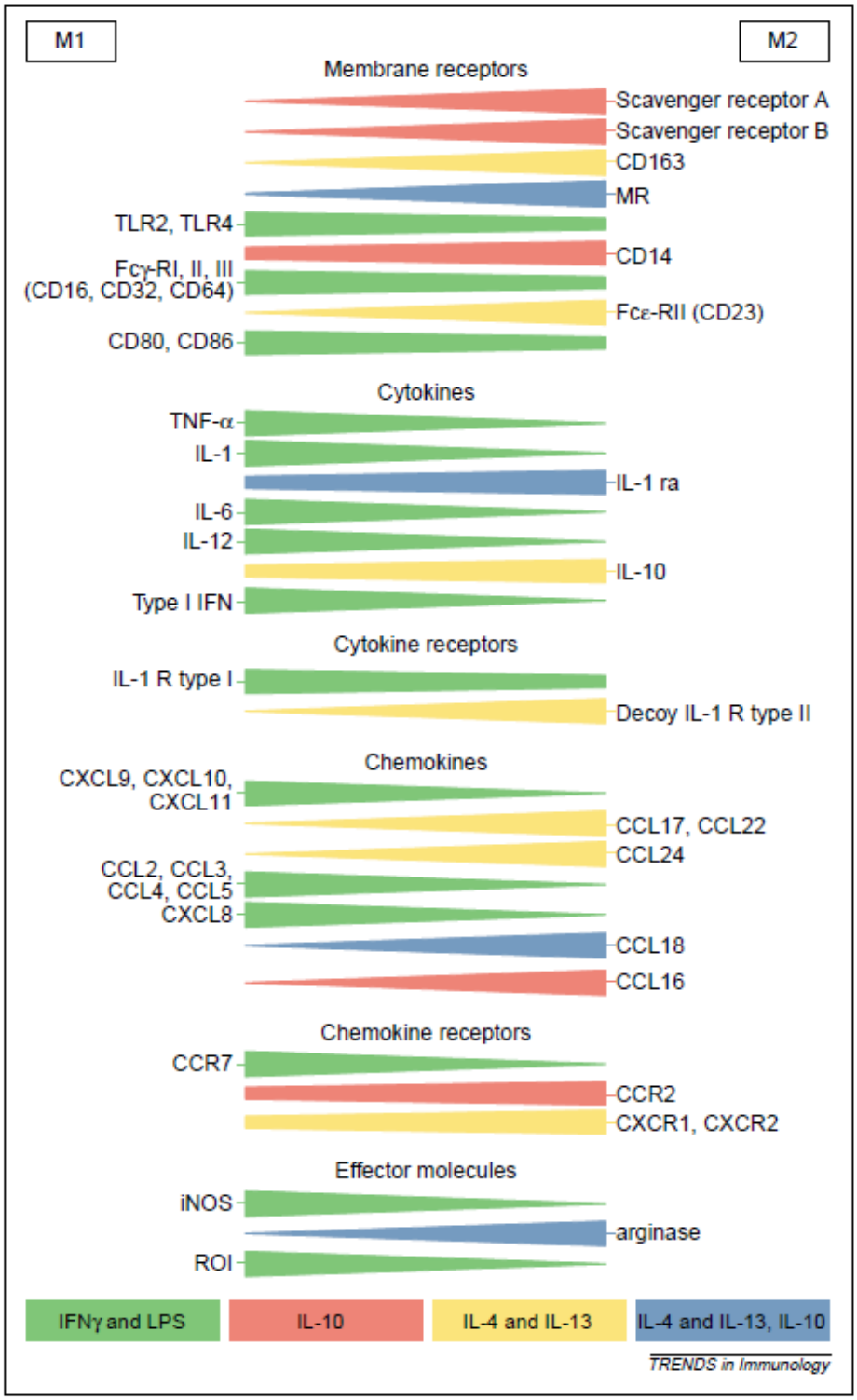

M1
M2
Membrane receptors
Scavenger receptor A
Scavenger receptor B
CD163
MR
TLR2, TLR4
CD14
FcγRI, II, III
(CD16, CD32, CD64)
FcεRII (CD23)
CD80, CD86
Cytokines
TNF-α
IL-1
IL-1 ra
IL-6
IL-12
IL-10
Type I IFN
Cytokine receptors
IL-1 R type I
Decoy IL-1 R type II
Chemokines
CXCL9, CXCL10,
CXCL11
CCL17, CCL22
CCL24
CCL2, CCL3,
CCL4, CCL5
CXCL8
CCL18
CCL16
Chemokine receptors
CCR7
CCR2
CXCR1, CXCR2
Effector molecules
iNOS
arginase
ROI
IFNγ and LPS
IL-10
IL-4 and IL-13
IL-4 and IL-13, IL-10
TRENDS in Immunology

**Figure 1.3. "M1 and M2 macrophages, the extremes of a continuum"**

From: Mantovani *et al*.. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; 23(11): 549-55.

Despite this wealth of knowledge regarding macrophage activation states there remain many unanswered questions. Molecular mechanisms underlying macrophage activation states remain largely undefined. Tuning of nuclear factor-κB (NF-κB) activation by p50 homodimers has been shown to be associated with M2 differentiation (Biswas *et al*., 2006). Peroxisome proliferator-activated receptor-γ (PPARγ) agonists are known to induce M2-like differentiation (Bouhlel *et al*., 2007) and the phosphatase SH2 domain-containing inositol 5'-phosphatase (SHIP) has been shown to play a key role in balancing macrophage activation, although recent evidence suggests that it acts indirectly through the regulation of IL-4 production by basophils (Kuroda *et al*., 2009). Ishii and colleagues recently identified a molecular pathway responsible for epigenetic regulation of important murine M2-associated genes (Ishii *et al*., 2009). IL-4 up-regulates the histone demethylase Jmjd3 via the transcription factor STAT6. Jmjd3 contributes to demethylation of histone H3 at lysine 27, thus unleashing promoters of M2 marker genes (*arginase 1, Ym1, mannose receptor* and *FIZZ-1*). These findings offer a novel mechanistic insight into macrophage activation and may pave the way to its manipulation and targeting.

**Macrophages and tissue fibrosis**

It is well recognised that monocytes and macrophages are important modulators of fibrosis in other organs. Duffield and colleagues highlighted the importance of macrophages in the pathogenesis of liver fibrosis (Duffield *et al*., 2005a). Using the CD11b-DTR transgenic mouse in which administration of diphtheria toxin (DT) conditionally and specifically depletes macrophages (as will be discussed in more detail later), they demonstrated that depletion of macrophages during liver fibrogenesis reduced fibrosis as quantified by sirius red staining and immunohistochemistry for collagen III and α-SMA. In contrast, depletion of macrophages during the resolution phase of experimental liver fibrosis demonstrated that macrophages were required for this resolution as animals treated with DT showed a slowing of resolution as quantified

by sirius red staining and immunohistochemistry for collagen III and α-SMA. Work from the same group has since established that scar associated macrophages are the major source of MMP13 which is responsible in part for the resolution of liver fibrosis (Fallowfield *et al*., 2007). Research from another group has recently established the importance of the $Ly6^{hi}$ population of monocytes in the pathogenesis of liver fibrosis (Karlmark *et al*., 2009). They showed that $Ly6^{hi}$ monocytes are massively recruited into the liver upon liver fibrogenesis, as induced by carbon tetrachloride, and can be identified by their $CD11b^{+}F4/80^{+}Ly6C^{hi}$ phenotype. In addition, liver fibrosis was exacerbated following adoptive transfer of bone-marrow derived $Ly6C^{hi}$ monocytes and CCR2-null mice had reduced liver fibrosis, suggesting that CCR2-mediated recruitment of $Ly6C^{hi}$ monocytes was crucial to the development of liver fibrosis.

Having established the importance of macrophages in liver fibrosis, Duffield and colleagues went on to assess the importance of macrophages in renal fibrosis (Duffield *et al*., 2005b). Using CD11b-DTR transgenic mice they established that depletion of macrophages reduced kidney injury as assessed histologically and by analysis of renal function. Furthermore immunohistochemical analysis revealed a reduction in collagen III and α-SMA staining following macrophage depletion, and a reduction in both myofibroblast proliferation and apoptosis, suggesting that macrophages support a population of renal myofibroblasts in a highly dynamic state during renal fibrogenesis. Duffield's group has since gone on to show that $Ly6^{hi}$ monocytes are recruited to the kidney during fibrogenesis and are important for promoting fibrosis (Lin *et al*., 2009).

**Macrophages and pulmonary fibrosis**

Previous studies have attempted to ascertain the role of macrophages in lung fibrosis but they have all used models with questionable direct relevance to lung fibrosis (Zhang-Hoover *et al*., 2000; Misson *et al*., 2004; Bem *et al*., 2004).

Interest in the role of macrophages in lung fibrosis is not new. Several papers in the 1980s highlighted their potential importance (Rennard *et al*., 1981; Bitterman *et al*., 1983; Bitterman *et al*., 1984; Lacronique et al., 1984; Bitterman et al., 1986; Rennard *et al*., 1988; Nagaoka *et al*., 1990). Bitterman and colleagues (Bitterman *et al*., 1983) demonstrated that alveolar macrophages from patients with IPF release a soluble factor that stimulates fibroblast growth and proliferation. They cultured non-cycling fibroblasts with the supernatants of unstimulated macrophages from patients with IPF and normal controls and discovered that there was a marked increase in fibroblast replication rate when the source of the supernatants were patients with IPF. Interestingly this growth factor, which they initially termed alveolar macrophage derived growth factor (AMDGF), was subsequently discovered to be IGF-1 (Rom *et al*., 1988). Work from the same group demonstrated that fibronectin was produced by human alveolar macrophages, and that the rate of production of fibronectin was ten times greater in patients with IPF compared to normal controls (Rennard *et al*., 1981). In addition they established that this macrophage-derived fibronectin was chemotactic for fibroblasts. Bitterman and colleagues then showed using a variety of techniques that alveolar macrophage replication is increased in patients with IPF as compared with controls and suggested that this expansion of mononuclear phagocytes may be important in effecting the surrounding molecular and cellular microenvironment (Bitterman *et al*., 1984).

At the time of these studies there was, as there still is now, debate about the role of macrophages in pulmonary fibrosis. Much of the uncertainty arises as a result of the lack of benefit of corticosteroids in treating patients with IPF (Turner-Warwick 1980b). Lacronique and colleagues demonstrated that corticosteroids do not affect the secretion of either fibronectin or AMDGF (IGF-1) by alveolar macrophages in normal patients or patients with IPF (Lacronique *et al*., 1984). Furthermore they established that this effect was not due to a lack of corticosteroid receptors, as patients with IPF bound corticosteroids similarly to normal volunteers. One other growth factor secreted by alveolar macrophages that has been investigated is PDGF. There are two human PDGF

genes, A and B (Collins *et al*., 1985; Rorsman *et al*., 1988). Martinet and colleagues discovered that alveolar macrophages from patients with IPF spontaneously released four times as much PDGF as alveolar macrophages from normal controls (Martinet *et al*., 1987). In addition PDGF was found to be chemotactic for smooth muscle cells and acted as a "competence factor for fibroblast growth". Subsequently it was shown that there is up-regulation in gene expression of *PDGF A* and *B* in patients with IPF as compared to normal controls (Nagaoka *et al*., 1990).

The first paper suggesting that an alternatively activated type of macrophage may be important in the pathogenesis of pulmonary fibrosis was published by Ann Millar's group (Hancock *et al*., 1998). They performed RT-PCR for IL-13 on RNA extracted from alveolar macrophages of 13 patients with various forms of fibrotic lung disease and 8 healthy controls. They also performed enzyme-linked immunosorbent assay (ELISA) for IL-13 on BALF, and culture supernatants from alveolar macrophages treated with LPS. They established that IL-13 message and protein were increased in patients with fibrotic lung disease, and moreover IL-13 production by alveolar macrophages cultured in the presence of LPS was increased in patients with fibrotic lung disease compared to controls.

However research in this area has not progressed, in part as a consequence of the lack of benefit of corticosteroids in treating fibrotic lung diseases. As discussed previously, investigation into the pathogenesis of pulmonary fibrosis has focused on the aberrant wound healing model (Selman *et al*., 2001; Selman *et al*., 2006). This hypothesis however does not take into account the natural history of the disease but rather reflects a single "snapshot" view, and does not recognise the fact that injury to any tissue is often followed by an inflammatory response and subsequent repair. While a poor response to conventional anti-inflammatory therapy at the end stage of fibrosis has been cited in support of this hypothesis, this negative finding should not rule out the possibility of a pathogenic role for early-stage inflammation. Zuo and colleagues (Zuo *et al*., 2002) used oligonucleotide microarray analysis to compare gene expression patterns in lung

samples from patients with histologically proven IPF to those of healthy control subjects. The expression of four categories of genes was markedly increased. These included genes that were associated with cell contraction, including those that encode smooth muscle proteins. Particularly up-regulated contractility genes included *actin*, *myosin*, and *tropomyosin*. The expression of genes that encode proteins involved in signalling (cell adhesion kinase), extra-cellular matrix (ECM) formation (collagen I and III, fibronectin), and ECM degradation (MMP-1, MMP-2, MMP-7, and MMP-9) were also increased. Interestingly and importantly, a third set of genes demonstrated the expression of a number of pro-inflammatory cytokines, chemokines, and antioxidants. Furthermore, there was up-regulation in expression of a fourth set of genes, encoding amyloid and immunoglobulins, genes that would normally be associated with chronic inflammatory disorders.

Another study by Hunninghake and colleagues (Hunninghake *et al*., 2003) which was designed to identify clinical and radiologic findings associated with a pathologic diagnosis of UIP, found that chest radiographic findings consistent with UIP and two high-resolution CT scan findings (lower lobe honeycombing and irregular lines in the upper lobes) were positively associated with a diagnosis of UIP. Unexpectedly, there was significant mediastinal adenopathy in the majority (55%) of patients with a diagnosis of IPF. This finding may suggest that the majority of IPF patients have an ongoing immune lympho-proliferative process, presumably in response to some unknown antigen. Taken together, the studies of Zuo and colleagues and Hunninghake and colleagues demonstrate that inflammatory-associated genes are up-regulated in patients with IPF and that the host responds with immune-mediated lympho-proliferation. An interesting study by Flaherty and colleagues (Flaherty *et al*., 2001b) sheds some light on the natural history of UIP and may hint at the association between inflammation and UIP. This prospective study evaluated multiple open lung biopsies involving more than one lobe of the lung in 109 patients with UIP. Forty-seven percent of patients (mean age 63.3 years) exhibited the histopathology of UIP in all lobes. Twenty-six percent of patients (mean age 56.9 years) demonstrated the histopathology

of non-specific interstitial pneumonia (NSIP) in at least one lobe and UIP in at least one lobe, indicating the presence of two different idiopathic pulmonary processes in the lung at the same time. NSIP is believed to be an inflammatory condition, which in the majority of cases is responsive to corticosteroids (Travis *et al*., 2008). In the remaining 26% of patients (mean age 53.1 years), NSIP was found in all lobes. Ten percent of patients had two or more biopsy specimens obtained from the same lobe; among these patients, 73% of the lobes had co-existent NSIP and UIP.

Importantly, it has been demonstrated that corticosteroids actually induce alternative activation of macrophages (Gratchev *et al*., 2001; Gratchev *et al*., 2005), and several recent studies have suggested that alternatively activated macrophages may regulate myofibroblasts *in vitro* (Song *et al*., 2000; Prasse *et al*., 2006). In some studies macrophages with pro-fibrotic functions have been implicated as key effector cells in the pathogenesis of IPF (Uh *et al*., 1998; Wynes *et al*., 2003; Wynes *et al*., 2004). Several other papers have suggested the potential importance of macrophages (Mora *et al*., 2006; Prasse *et al*., 2006; Okuma *et al*., 2004; Baran *et al*., 2007).

Okuma and colleagues examined the pathogenesis of pulmonary fibrosis using the bleomycin model (Okuma *et al*., 2004). They established at 21 days post-bleomycin administration that CCR2-null mice showed limited areas of fibrosis and retained alveolar structures as visualised by histology in contrast to CCR2 wild-type mice which exhibited diffuse fibrosis and obliteration of normal alveolar structures. Reduction in fibrosis in CCR2-null mice was corroborated by hydroxyproline assay. Analysis of differential cell counts from BALF demonstrated a peak influx of macrophages between days 14 and 21, with a significant reduction in numbers of BALF macrophages in the CCR2-null mice. Interestingly, they showed that BALF activities of MMP2 and MMP9 were reduced at day 14 in CCR2-null mice, that MMP2 and MMP9 activity in proteins extracted from alveolar macrophages were reduced at day 3 in CCR2-null mice, and that MMP2 and MMP9 staining as visualised by immunocytochemistry was reduced at day 3

in CCR2-null mice. It should be noted that in their model peak fibrosis occurs around 21 days, and as such the specifics of the MMP results should be interpreted with caution. In a murine model of pulmonary fibrosis induced by the administration of murine γ-herpes-virus 68 to IFN-γ-receptor-deficient mice, Mora and colleagues (Mora *et al*., 2006) demonstrated that there was recruitment of alveolar macrophages to areas of epithelial hyperplasia and fibrosis. Furthermore these macrophages showed expression of molecules (such as Ym1 and arginase I) indicative of activation of macrophages by the alternative pathway. As alternatively activated macrophages are associated with tissue fibrosis, the authors suggested that alternative activation of macrophages may be important in the pathogenesis of pulmonary fibrosis.

Using the bleomycin model of pulmonary fibrosis Baran and colleagues (Baran *et al*., 2007) showed that macrophage colony-stimulating factor (M-CSF)- and CCL2- null mice had less lung fibrosis, mononuclear phagocyte recruitment, collagen deposition, and CTGF expression than wild type. In addition they found that BALF from patients with IPF contained significantly more M-CSF and CCL2 than BALF from normal volunteers, suggesting an important role for M-CSF and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis.

The importance of a human marker of alternative macrophage activation, CCL18, has been suggested in two recent studies. Prasse and colleagues demonstrated that CCL18 mRNA and protein expression as visualised by *in-situ* hybridisation and immunohistochemistry, respectively, were increased in patients with IPF compared to controls, the increased expression being particularly obvious on epithelial cells (Prasse *et al*., 2006). Furthermore CCL18 gene expression and protein production were up-regulated in normal alveolar macrophages after co-culture with human lung fibroblasts, and they established that native collagen up-regulated CCL18 expression in normal alveolar macrophages that were alternatively activated. On the basis of these findings, the authors proposed that alveolar macrophages and fibroblasts may promote pulmonary fibrosis via the effects of CCL18. Interestingly they demonstrated that levels of CCL18

in BALF were higher in patients with IPF than controls, and correlated negatively with $DL_{CO}$. The same group subsequently showed that CCL18 is abundantly expressed in the culture supernatants of BALF cells, BALF and sera from patients with histologically proven pulmonary fibrosis (in a group of patients with a variety of interstitial lung diseases) and that there was a negative correlation between changes in total lung capacity and changes in serum CCL18 concentrations (Prasse *et al*., 2007). Specifically in relation to IPF they have since demonstrated that CCL18 serum concentrations predict outcome in IPF: using receiver operating characteristics analysis they found that there was a significantly higher mortality in patients with IPF in whom the serum CCL18 concentration was greater than 150ng/ml, and this corresponded with a higher incidence of disease progression (Prasse *et al*., 2009). CCL18, also known as AMAC1 (alternative macrophage activation-associated CC-chemokine 1) is a marker of alternative macrophage activation (Gordon 2002). It has been suggested to share some features with TGFβ (Prasse *et al*., 2009) as like TGFβ it stimulates collagen production in lung fibroblasts (Atamas *et al*., 2003).

These studies provide circumstantial evidence for the putative role of macrophages in the pathogenesis of pulmonary fibrosis and highlight the need for further studies to confirm and further define the role of macrophages in pulmonary fibrosis.

**Circulating monocytes**

The kinetics and origin of monocytes were first described by van Furth and Cohn (van Furth & Cohn 1968; van Furth *et al*., 1970). Using tritiated-thymidine labelling of monocytes, they showed that monocytes in the peripheral blood do not multiply, but that mononuclear phagocytes of the bone marrow do and are the progenitors of blood monocytes. In addition, following injection of newborn calf serum into the peritoneum they established that there was a rapid extravasation of blood monocytes into the

peritoneal cavity, and they concluded that monocytes in the peripheral blood are the precursors of tissue macrophages (van Furth & Cohn 1968).

Mouse monocytes were originally phenotypically defined as cells expressing high levels of CD11b, Ly6C and CD62L (Lagasse & Weissman 1996), and not expressing CD11c nor MHC class II molecules (Leon *et al*., 2004). Jung and colleagues developed an CX3CR1GFP/+ transgenic mouse where one allele for the gene encoding CX3CR1, the receptor for fractalkine, has been replaced by the gene encoding GFP. This results in GFP labelling of all circulating $CD11b^{+}F4/80^{+}$ cells (Jung *et al*., 2000). Using this system Geissmann and colleagues (Geissmann *et al*., 2003) defined two murine monocyte subsets: $CX3CR1^{lo}$ monocytes which are large granular mononuclear cells, and $CX3CR1^{hi}$ monocytes which are small mononuclear cells. Using Ki67 they confirmed the findings of van Furth & Cohn that monocytes are non-cycling in the blood. Flow cytometric analysis of the subsets further defined the cells as $CX3CR1^{lo}$, $CCR2^{+}$, $CD62L^{+}$, $Gr1^{+}$ and $CX3CR1^{hi}$, $CCR2^{-}$, $CD62L^{-}$, $Gr1^{-}$, respectively. However, the Gr1 label refers to the specificity of the monoclonal antibody RB6-8C5 that recognises both the Ly6G and Ly6C antigens (Fleming *et al*., 1993), and as Ly6G is expressed only on granulocytes (Fleming *et al*., 1993) whereas Ly6C is expressed on both monocytes (Sunderkötter *et al*., 2004) and granulocytes (Fleming *et al*., 1993), it was felt that the Gr1 label was not appropriate for classifying monocytes. As such, the subsets are more correctly identified as $CX3CR1^{lo}$, $CCR2^{+}$, $CD62L^{+}$, $Ly6C^{hi}$ and $CX3CR1^{hi}$, $CCR2^{-}$, $CD62L^{-}$, $Ly6C^{lo}$, respectively (Auffray *et al*., 2009) (Figure 1.4).
Geissmann and colleagues (Geissmann *et al*., 2003) went on to stain human peripheral blood cells with a fractalkine-Fc fusion protein specific for CX3CR1 and identified by flow cytometry that there were two major subsets of monocytes: $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$. These subsets, although differing from murine monocytes in terms of CD11c and MHC II expression, have been paired with the murine monocyte subsets $CX3CR1^{lo}$, $CCR2^{+}$, $CD62L^{+}$, $Ly6C^{hi}$ and $CX3CR1^{hi}$, $CCR2^{-}$, $CD62L^{-}$, $Ly6C^{lo}$, respectively. They have in common size, granularity, and expression patterns of

adhesion molecules and chemokines receptors. The Ly6C$^{hi}$ subset is often referred to as the "inflammatory" monocyte (Gordon & Taylor 2005; Tacke & Randolph 2006).

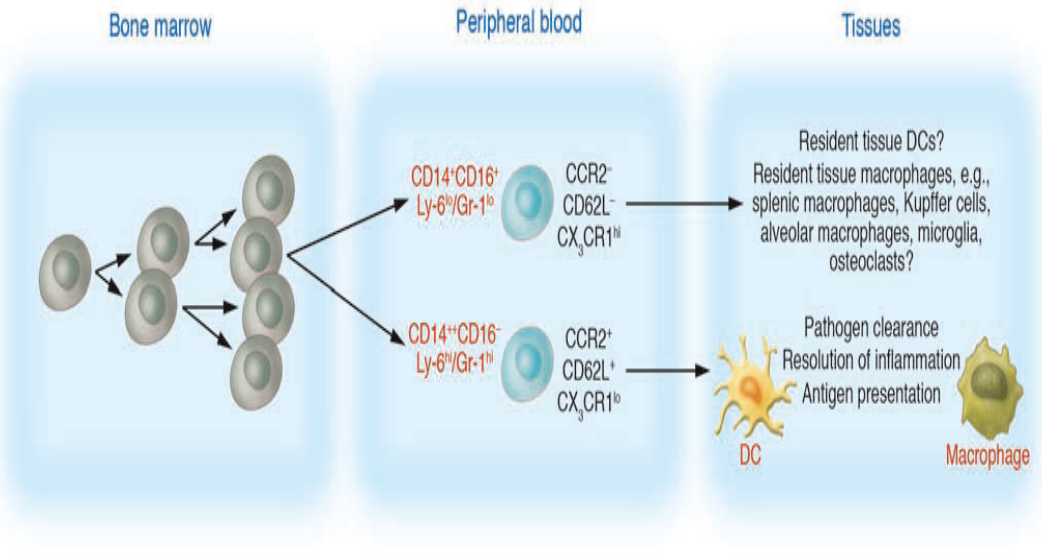



**Figure 1.4. Blood monocyte populations.**

From: Gordon S. Macrophage heterogeneity and tissue lipids. *J Clin Invest* 2007; 117(1): 89-93.

**Monocytes, fibrocytes and pulmonary fibrosis**

The role of monocytes in fibrosis has recently been intensely studied. As discussed earlier, Karlmark and colleagues have recently shown in the context of liver inflammation and fibrosis that inflammatory Ly6C$^{hi}$ monocytes are recruited into the injured liver via CCR2-dependent bone marrow egress, and promote the progression of liver fibrosis (Karlmark *et al*., 2009). In addition, Lin and colleagues have recently shown that Ly6C$^{hi}$ monocytes are recruited to the kidney following unilateral ureteric kidney obstruction, a model for renal fibrosis, and differentiate into monocyte populations that participate in the promotion of kidney fibrosis (Lin *et al*., 2009)

Until very recently there has been very little interest in the role of monocytes in lung fibrosis. Much of the interest in circulating cells that contribute to lung fibrosis has centred on the fibrocyte. As mentioned previously, fibrocytes were first described by Bucala and colleagues (Bucala *et al*., 1994). These cells were CD45$^{+}$, CD34$^{+}$, ColI$^{+}$, but in addition were also positive for the markers vimentin, fibronectin, collagen III, CD11b and CD18. Fibrocytes were found in regions of scar formation and it was proposed by Bucala and colleagues that these cells may play a role in a variety of pathological conditions characterised by excessive fibrosis. They were subsequently found to comprise only 0.1-0.5% of nucleated cells in peripheral blood (Quan *et al*., 2004). Phillips and colleagues established that human fibrocytes express CD45, Col I and CXCR4 and migrate in response to CXCL12 (Phillips *et al*., 2004). They went on to establish that fibrocytes accumulate in the lungs of bleomycin-treated mice, and that administration of specific neutralising anti-CXCL12 (the ligand for CXCR4) antibodies diminished the recruitment of fibrocytes to the lung and ameliorated lung fibrosis as measured by Sircol assay. Moeller and colleagues sampled blood from patients with stable IPF and during acute exacerbations of the disease (Moeller *et al*., 2009). Fibrocytes were defined by flow cytometry as CD45$^{+}$Col I$^{+}$. They established that there was an increase in numbers of fibrocytes in patients with stable IPF compared with healthy controls, and that the numbers were further increased in patients during acute

exacerbations. Fibrocyte numbers were not correlated with radiological severity scores or lung function, but were in fact an independent predictor of mortality; the mean survival of patients with a fibrocyte proportion of greater than 5% of total blood leukocytes was only 7.5 months, in contrast to 27 months for patients with a fibrocyte proportion of less than 5% (Moeller *et al*., 2009).

The potential link between fibrocytes and monocytes has recently been highlighted in an elegant paper by Niedermeier and colleagues (Niedermeier *et al*., 2009). Using cell depletion experiments and purified cells they demonstrated that $CD115^+$, $CD11b^+$, $Ly6C^{hi}$ monocytes appear to be the precursor cells of murine fibrocytes, but require $CD4^+$ T cells or molecules derived from them to make this transition. Interestingly, activation of $CD4^+$ T cells in the presence of cyclosporin A markedly enhanced the production of fibrocytes *in vitro* and increased the level of *Collagen I* mRNA and tissue fibrosis in the unilateral ureteric obstruction model of renal fibrosis. Treatment with rapamycin in contrast did not affect the number of fibrocytes, expression of Collagen I or tissue fibrosis.

Two papers just published have provided more direct evidence for a role of monocytes in the pathogenesis of pulmonary fibrosis. Murray and colleagues analysed plasma from IPF and control patients and showed that the two monocyte markers IL-1RA and CCL2 (MCP-1) were increased in patients with IPF (Murray *et al*., 2010). In addition they demonstrated that patients with "progressive" IPF had a greater percentage of $CD14^+$ monocytes expressing the human alternatively activated macrophage marker CD163 compared with patients with stable IPF and controls. Analysis of protein lysates of whole lung tissue showed that there were elevated levels of the M2 macrophage markers IL-13 and IL-1RA in patients with IPF, but not IL-4 or resistin. The level of the M1 macrophage marker $CX_3CL1$ was decreased in patients with IPF, but there was no reduction in the levels of other M1 markers CXCL10/IP10 and CCL3/MIP-1$\alpha$. They went on to show that in mice treated with bleomycin to induce pulmonary fibrosis that additional administration of serum amyloid P (SAP) on days 11-20 of their 21 day

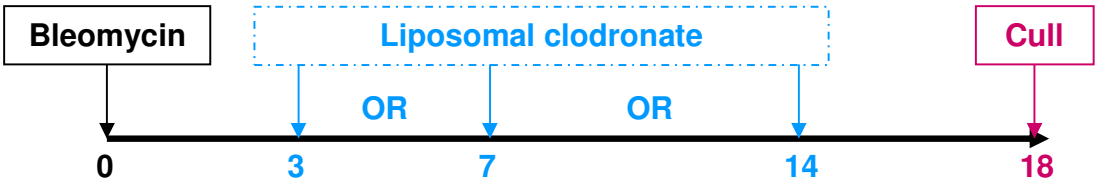
model resulted in a reduction in fibrosis, as visualised on lung sections stained with haematoxylin and eosin (H&E), and quantified by hydroxyproline assay. The authors concluded that SAP-mediated inhibition of alternatively activated macrophages may be a therapeutic strategy for patients with IPF. Work from the same group has shown that blood from patients with systemic sclerosis-interstitial lung disease is enriched for fibrocytes, $CD14^+$ monocytes, $CD163^+$ monocytes as well as IL-4, IL-10, IL-13, CCL2 and CCL18, suggesting that alternatively activated macrophages may be important in the pathogenesis of fibrotic lung disease (Mathai *et al*., 2010).

**Aims and hypothesis**

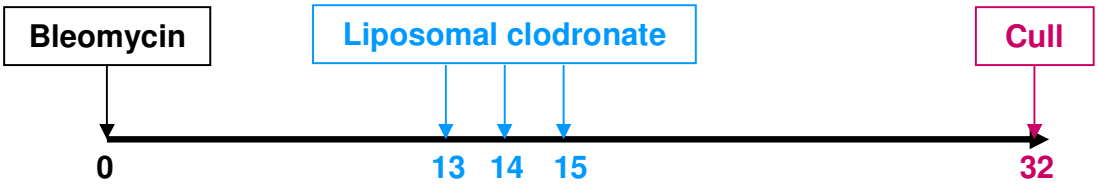
As can be seen from the introduction above, it is clear that while great advances have been made in understanding the pathogenesis of pulmonary fibrosis, there remains much controversy. In particular, the role of "immune-type" cells is still uncertain. With this mind, and taking into account previous work exploring the role of macrophages in pulmonary fibrosis, I hypothesised that monocytes and macrophages would be important in regulating pulmonary fibrosis.

As such, the aim of my research is to establish categorically the role of monocytes and macrophages in pulmonary fibrosis. I aim to establish whether circulating monocytes and lung macrophages are important during various phases of pulmonary inflammation and fibrosis, using the best available models of pulmonary fibrosis, bleomycin and AdTGFβ, and several methods of cellular depletion. The experimental outlines for this are described below:

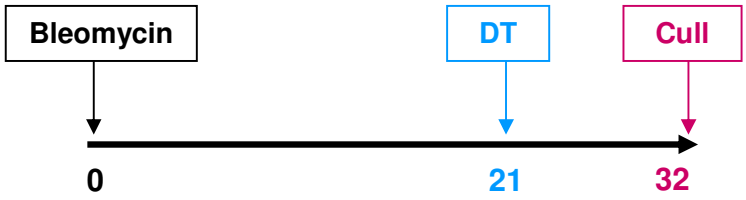
i.) Are lung macrophages important during the inflammation initiation of pulmonary fibrosis?




ii.) Does depletion of lung macrophages during the inflammatory phase of bleomycin-induced pulmonary fibrosis have any effect on subsequent pulmonary fibrosis?




iii.) Do lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model?



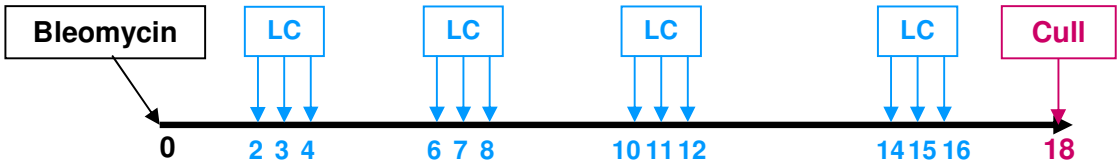


DT=diphtheria toxin (CD11c:DTR mouse)

iv.) Do lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis in the AdTGFβ model?

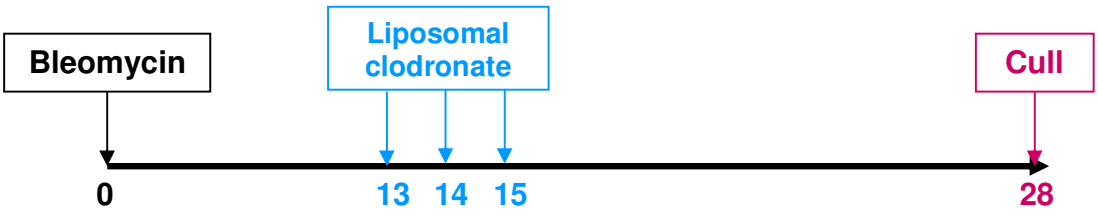

v.) Do lung macrophages regulate the resolution phase of pulmonary fibrosis in the bleomycin model?

vi.) Are circulating monocytes important during the inflammation initiation of pulmonary fibrosis?

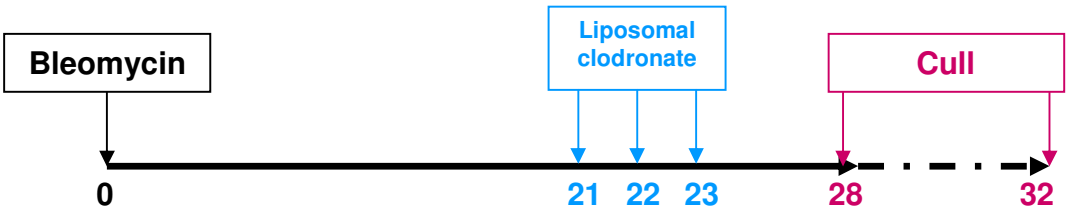


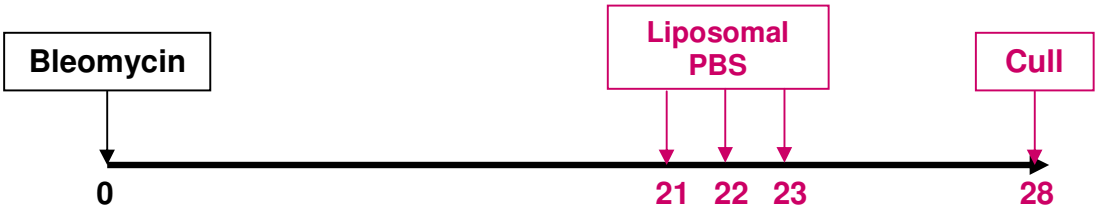

LC=liposomal clodronate

vii.) Does depletion of circulating monocytes during the inflammatory phase of bleomycin-induced pulmonary fibrosis have any effect on subsequent pulmonary fibrosis?

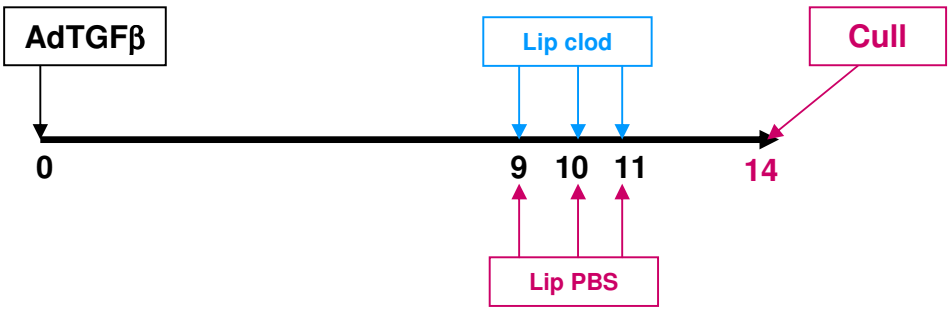



viii.) Do circulating monocytes regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model?






ix.) Do circulating monocytes regulate the progressive fibrotic phase of pulmonary fibrosis in the AdTGFβ model?




I plan to confirm any significant effect of depletion using a complimentary add-back or add-in experiment. By focusing on MMP biology, I aim to establish whether new mechanistic insights into pulmonary fibrosis pathogenesis can be gained. Finally, by using human tissue samples, I hope to corroborate any findings identified in the mouse, and as such provide translational evidence for the potential importance of any novel results that may be identified.

# CHAPTER 2

## MATERIALS & METHODS

### *2.1 Animals*

Female C57Bl/6 mice were bred in-house or purchased from Harlan and maintained in 12-hour light/12-hour dark cycles with free access to food and water. All procedures were performed in accordance with Home Office guidelines [Animals (Scientific Procedures) Act 1986].

### *2.2 Bleomycin model of pulmonary fibrosis*

Female mice 8-14 weeks old were anaesthetised with isoflurane (L2901, Merial Animal Health Ltd, Harlow, UK), and bleomycin sulphate (BI3543, Apollo Scientific, Stockport, UK) (0.0167mg or 0.033 mg in 50 µl of 0.9% saline) or 0.9% saline was administered intra-tracheally by an aspirational technique. For comparative studies mice were age- and weight-matched. Mice were culled on day 3, 7, 14, 18, 21, 22, 25, 28, 32, 56 and 136 by intra-peritoneal injection of pentobarbital sodium (Merial Animal Health Ltd, Harlow, UK). Lungs were removed en bloc; the left lobe was tied off and snap frozen in liquid nitrogen for collagen analysis. In some experiments, in addition, the right apical lobe was tied off and snap frozen in liquid nitrogen for RNA/protein analysis. In some experiments the trachea was cannulated and the lungs lavaged with 3 x 0.8 ml (4 x 0.8ml for flow cytometry experiments) sterile Dulbecco's phosphate buffered saline (PBS) pH7.4 (D8537, Sigma, UK) containing 2mM EDTA (E5134, Sigma, UK). The remaining lung was inflated under gravity with methyl carnoys solution (60% methanol (C-M400017-X, Fisher Scientific, UK), 30% chloroform (C-C496017-X, Fisher Scientific, UK), 10% glacial acetic acid (A6283, Sigma, UK)) or 10% formalin solution (Sigma, UK) and then incubated in the same solution overnight.

Lungs were then transferred into 70% ethanol prior to paraffin embedding and sectioning for histology and immunohistochemistry.

*2.3 AdTGFβ model of pulmonary fibrosis*

AdTGFβ refers to porcine TGF-$\beta_1$ adenovirus (AdTGF$\beta1^{223/225}$), an adenovirus construct containing a mutation of cysteine to serine at positions 223 and 225, rendering the expressed TGF$\beta_1$ biologically active (Sime *et al*., 1997). Control virus (AdDL70-3) was constructed as previously described (Bett *et al*., 1994). Both AdTGFβ and AdDL70-3 were kindly supplied by Professor Jack Gauldie (Hamilton, Ontario). Female mice 10-14 weeks old, weight-matched, were anaesthetised with isoflurane, and AdTGFβ or AdDL70-3 administered intra-tracheally (50μl). Mice were culled on days 5 and 14 by intra-peritoneal injection of pentobarbital sodium (Merial Animal Health Ltd, Harlow, UK). Retrieval and processing thereafter was performed as described above for the bleomycin model.

*2.4 Liposomal clodronate and liposomal PBS*

Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. It was encapsulated in liposomes as previously described (van Rooijen & Sanders 1994). Liposomal PBS was supplied by Dr Nico van Rooijen (Amsterdam, Netherlands). Liposomal clodronate and liposomal PBS were administered intra-tracheally (100μl) or intra-peritoneally (400μl). For intra-tracheal administration mice were anaesthetised with isoflurane and the liposomal clodronate given by aspirational technique as for bleomycin and AdTGFβ.

*2.5 Diphtheria Toxin*

Diphtheria toxin (D0564, Sigma, UK) was re-constituted in sterile $H_2O$ at a concentration of 1mg/ml and stored at -80$^{o}$C. Upon thawing of aliquots, DT suspension was diluted in sterile PBS to make a working stock solution at a concentration of 100µg/ml.

*2.6 Determination of lung fibrosis*

Paraffin-embedded sections of mouse tissue were stained with Masson's trichrome as per manufacturer's instructions. Fibrosis score was evaluated (x100) as the quantity of the section positively stained for collagen and displaying alveolar wall thickening (1 = <25%, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%). This score is as yet unpublished. It was developed by Professor Sarah Howie (MRC/University of Edinburgh Centre for Inflammation Research, Edinburgh), as a modified version of the Ashcroft Score (Ashcroft *et al*., 1988), that is felt to be more applicable to the bleomycin model, combined with a previously published fibrosis score used in the bleomycin model (Pardo *et al*., 2003). Professor Howie ensured that I was adequately trained. Only fields where the majority of the field was composed of alveoli were scored. Blinded analysis of the *entire* lung section was performed. Mulitple practice runs were performed until scoring became consistent. Thereafter all lung sections were scored with no time gaps to minimise variability of scoring. Repeat scoring ensured that the results were reproducible.

*2.7 Determination of lung inflammation*

Paraffin-embedded sections of mouse tissue were stained with Masson's trichrome as per manufacturer's instructions. Peri-vascular and peri-bronchial inflammation scores were evaluated based on methods previously published (Fisher *et al*., 2005), with minor modifications to ensure applicability to the bleomycin model. Peri-vascular

inflammation score was evaluated (x100) as the number of cells surrounding vessels (1 = <10, 2 = 10-20, 3 = 20-50, 4 = >50) that were <50% of the size of the field in view. Peri-bronchial inflammation score was evaluated (x100) as the number of cells surrounding bronchi (1 = <10, 2 = 10-20, 3 = 20-50, 4 = >50) that were <50% of the size of the field in view. Only fields where the majority of the field was composed of alveoli were scored. A run-in period and confirmatory scoring were performed as for the lung fibrosis score (above). Blinded analysis of the *entire* lung section was performed.

*2.8 Determination of lung collagen by Sircol assay*

Collagen content of the left lung was carried out by Sircol assay as per manufacturer's instructions (Biocolor Ltd, County Antrim, UK); the left lung was minced in 5 ml of 0.5M acetic acid (A6283, Sigma, UK) with pepsin (P6887, Sigma, UK) added at a ratio of 1:10 (w:w, pepsin:lung) and incubated with shaking at 4$^{o}$C for 24 hours. Cleared lung extract (0.2 ml) was incubated with 1ml Sircol dye reagent for 30 minutes on a rotator at room temperature then centrifuged at 12,000g for 10 minutes at 4$^{o}$C. Supernatant was decanted. Pellets were solubilised in 1 ml 0.5M sodium hydroxide (S5881, Sigma, UK) and absorbance measured at 540 nm alongside collagen standards (Sircol bovine collagen reference standard, S1010, Biocolor Ltd, County Antrim, UK).

*2.9 Immunohistochemistry*

Tissues were fixed with methyl carnoys solution and embedded in paraffin. 5µm sections were treated with 12.5% proteinase K [dissolved in pre-heated PBS] (P8044, Sigma, UK) for 5 minutes at 37$^{o}$C (F4/80) or microwaved in 10mM sodium citrate (S4641, Sigma, UK) for 15 minutes (α-SMA, Ym1) for antigen retrieval. Sections were blocked with 1% hydrogen peroxide [dissolved in de-ionised $H_20$] (H1009, Sigma, UK) for 15 minutes followed by 20% rabbit serum [diluted in PBS] (X0902, Dako UK Ltd, Cambridgeshire) (F4/80) or 20% swine serum [diluted in PBS] ((X0901, Dako UK Ltd, Cambridgeshire) (α-SMA, Ym1) for 30 minutes. Sections were then incubated with

primary antibody for 1hour to F4/80 (Rat monoclonal [CI:A3-1] anti-F4/80, 1:300, AB6640, Abcam, Cambridge, UK), α-SMA (Murine monoclonal anti-α-smooth muscle actin, clone 1A4, FITC conjugate, 1:15,000, F377, Sigma, UK) or Ym1 (Rabbit polyclonal anti-Ym1, 1:100, 01404, Stem cell technologies, Grenoble, France) followed by a biotinylated secondary antibody for 30 minutes (F4/80: biotinylated polyclonal rabbit anti-rat, 1:400, E0468, Dako UK Ltd, Cambridgeshire; α-SMA: polyclonal rabbit anti-FITC, 1:500, 71-1900, Zymed Laboratories Inc., USA; Ym1: biotinylated polyclonal swine anti-rabbit, 1:200, E0431, Dako UK Ltd, Cambridgeshire). In the case of α-SMA this was followed by a biotinylated tertiary antibody for 30 minutes (Biotinylated polyclonal swine anti-rabbit, E0353, Dako UK Ltd, Cambridgeshire). For all antibodies this was followed by addition of the ABC reagent (R.T.U. Vectastain Elite ABC Reagent, PK-7100, Vector Labs, Burlingame, CA, USA) for 30 minutes followed by liquid diaminobenzidine (DAB) (Liquid DAB+Substrate Chromogen System, K3468, Dako UK Ltd, Cambridgeshire) in the dark for 5 (α-SMA, Ym1) or 10 (F4/80) minutes. Sections were counterstained with haematoxylin, dehydrated in alcohols, and mounted. The following isotype controls were used: for F4/80: rat IgG (sc2026, Santa Cruz Biotechnology, USA) and for Ym1: rabbit IgG (sc2027, Santa Cruz Biotechnology, USA). Ym1 and F4/80-positive cells taken from >50 randomly selected high-power fields (x200 Ym1, x320 F4/80) (if more than 50 fields, 200 cells were used instead) were quantified.

It should be noted that αSMA is generally accepted to be a marker of myofibroblasts (Zhang *et al*., 1994). F4/80 is a murine macrophage restricted marker (Austyn & Gordon 1981), and Ym1 is an archetypal marker of alternative macrophage activation (Gordon 2003).

*2.10 Cytospins*

Cell suspensions were created in a solution of 0.1% bovine serum albumin (A7906, Sigma, UK). Numbers of cells were determined using nucleocassettes (941-0002,

Chemometec, Denmark) and a nucleocounter (SCC-100, Chemometec, Denmark). An aliquot of the cell suspension (20,000-40,000 cells) was pipetted into a cytofunnel (combined with a cytoclip, slide and filter card) (3120110, Thermo Scientific) and spun at 300RPM for 3 minutes using a Shandon Cytospin 4 cytocentrifuge (Thermo Scientific). Following air-drying slides were fixed and stained using Quik-Diff kit (Reagena, UK).

*2.11 Immunocytochemistry*

Cytospins were created as above using bronchoalveolar lavage fluid from mice and humans. However, instead of using the Quik Diff kit to fix and stain, slides were fixed in 90% anhydrous acetone/10% methanol (murine) or methanol (human)). Processing was as for immunohistochemistry except that no antigen retrieval was used. Processing using the Ym1 antibody used the same antibodies and controls as for IHC. For CD163 the following reagents were used: blocked with 20% rabbit serum [diluted in PBS] (X0902, Dako UK Ltd, Cambridgeshire); primary antibody: mouse monoclonal antibody [RM3/1] to CD163 (ab17051, 1:50, Abcam, Cambridge, UK); secondary antibody: biotinylated rabbit anti-mouse (E0354, 1:200, Dako UK Ltd, Cambridgeshire); isotype control antibody: mouse IgG (sc2025, Santa Cruz Biotechnology, USA).

*2.12 TGF$\beta_1$ ELISA*

TGF$\beta_1$ ELISA kit was purchased from R&D Systems (DuoSet ELISA development kit, DY 1679, Abingdon, UK). Capture antibody (mouse anti- TGF$\beta_1$, (from kit), 4µg per ml in Dulbecco's phosphate buffered saline (PBS) pH7.4 (D8537, Sigma, UK)) was added to 96-well microplates (Corning Costar cell culture plates (CLS3595, Sigma, UK) and incubated overnight at room temperature. Wells were aspirated and washed three times with wash buffer (0.05% Tween 20 (P2287, Sigma, UK) in Dulbecco's PBS). Plates were blocked by adding 300µl of block buffer (5% Tween 20 in Dulbecco's PBS) to each well and incubated at room temperature for 1 hour. Wells were aspirated and

washed three times with wash buffer. 100µl of sample (neat) or standards (Recombinant TGF$\beta_1$ (from kit), diluted in reagent diluent (1.4% bovine serum albumin (A7906, Sigma, UK) and 0.05% Tween 20 in Dulbecco's PBS, pH 7.2-7.5, 0.2µm filtered), working concentration 2000pg/ml, top standard 1000pg/ml) were added to wells and incubated for 2 hours at room temperature. Aspiration and wash were performed as above. 100µl of streptavidin-HRP (from kit) was added to each well and incubated for 20 minutes at room temperature. Aspiration and wash were performed as above. 100µl of substrate solution (3,3,5,5-tetramethylbenzidine liquid substrate (TMB, T4444, Sigma, UK)) was added to each well and incubated for 20 minutes at room temperature. 50µl of stop solution (2N $H_2SO_4$, DY994, R&D Systems, Abingdon, UK) was added to each well. The absorbance at 450nm was determined using a microplate reader (Synergy HT multi-mode microplate reader / Gen5 data analysis software, BioTek, UK), and TGF$\beta_1$ concentrations calculated from the standard curve.

*2.13 RNA extraction and qPCR*

Lung tissue was snap-frozen. Total RNA was extracted using an RNeasy Mini Kit (74104, QIAGEN, UK). First-strand cDNA synthesis was performed as outlined: (per reaction) 1 µg of total RNA (diluted to 9µl with $H_20$) added to 2µl random primers (50ng/µl, 48190-011, Invitrogen, UK) and heated at 70$^o$C for 10 minutes. This was followed by addition of 0.5µl SuperScript™ II Reverse Transcriptase (18064-014, Invitrogen, UK), 4µl 5X first strand buffer (Invitrogen, UK), 2µl 0.1M DTT, 0.5µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (10777019, Invitrogen, UK), 0.5µl of 10mM dNTP mix (U1240, Promega UK Ltd) and 1.5µl $H_2O$ and heated at 40$^o$C for 1 hour. cDNA was stored in aliquots diluted 1:100 at -20$^o$C prior to qPCR. For standards an identical volume of each sample was combined and the final mix diluted 1:10 to make the S1 standard. Subsequent standards (S1, S2, S3 *etc.*) were made by serial dilutions 1:1 with $H_2O$. qPCR was performed using SYBR-Green and TaqMan based detection methods (Invitrogen). For SYBR-Green based analyses the specificity of the

PCR products was confirmed by melting curve analysis. The standard curve method was used to quantify the expression of the target genes. The expression level of each gene was normalised to that of *18S* rRNA. *Ym1* was purchased as a TaqMan gene expression assay (MM00657889_MH, Applied Biosystems, UK). *18S* was purchased as a TaqMan endogenous control (4352930E, Applied Biosystems, UK). These primer products are predesigned and fully validated by Applied Biosystems. All other primers and probes were designed using primer express software. Their sequences are as follows:

SYBR

*α-SMA*: Forward 5'-TCAGCGCCTCCAGTTCCT-3'
Reverse 5'-AAAAAAAACCACGAGTAACAAATCAA-3'

*Col1A1*: Forward 5'-GATGACGTGCAATGCAATGAA-3'
Reverse 5'-CCCTCGACTCCTACATCTTCTGA-3'

*Arginase1*: Forward 5'-TTGGGTGGATGCTCACACTG-3'
Reverse 5'-TTGCCCATGCAGATTCCC-3'

*iNOS*: Forward 5'- CTATCTCCATTCTACTACTACCAGATCGA-3'
Reverse 5'- CCTGGGCCTCAGCTTCTCAT-3'

*MMP9*: Forward 5'- CGAACTTCGACACTGACAAGAAGT -3'
Reverse 5'- GCACGCTGGAATGATCTAAGC -3'

TaqMan

*MMP2*: Forward 5'- AACTACGATGATGACCGGAAGTG -3'
Reverse 5'- TGGCATGGCCGAACTCA -3'
Probe - TCTGTCCTGACCAAGGATATAGCCTATTCCTCG

*TIMP1*: Forward 5'- ATGGAAAGCCTCTGTGGATATG -3'
Reverse 5'- AAGCTGCAGGCACTGATGTG -3'
Probe - CTCATCACGGGCCGCCTAAGGAAC

*MMP13*: Forward 5'- GGGCTCTGAATGGTTATGACATTC -3'
Reverse 5'- AGCGCTCAGTCTCTTCACCTCTT -3'
Probe- AAGGTTATCCCAGAAAAATATCTGACCTGGGATTC

They have all been previously fully validated and optimised in the MRC/University of Edinburgh Centre for Inflammation Research by Dr Stephen Hartland, Iredale Group.

qPCR reaction mixtures were made as follows (per reaction): for SYBR-Green based analyses: cDNA 4µl, Express SYBR Green ER qPCR supermix (with premixed ROX) (11794, Invitrogen, UK) 5µl, forward primer 0.1µl, reverse primer 0.1µl, $H_2O$ 0.8µl. For TaqMan based analyses: cDNA 4µl, Express qPCR supermix (with premixed ROX) (11795, Invitrogen, UK) 5µl, forward primer 0.1µl, reverse primer 0.1µl, primer probe 0.1µl, $MgCl_2$ (Invitrogen, UK) 0.1µl, $H_2O$ 0.6µl. For Ym1: cDNA 4µl, Express qPCR supermix (with premixed ROX) (11795, Invitrogen, UK) 5µl, Ym1 TaqMan gene expression assay mix 0.5µl, $H_2O$ 0.5µl. For 18S: cDNA 0.8µl, Express qPCR supermix (with premixed ROX) (11795, Invitrogen, UK) 5µl, 18S TaqMan endogenous control mix 0.5µl, $H_2O$ 3.7µl. These reaction mixtures have been previously validated and optimised within the Forbes, Iredale and Sethi groups, MRC/University of Edinburgh Centre for Inflammation Research.

Samples were inserted into MicroAmp™ fast optical 96-Well reaction plates (4346906, Applied Biosystems, UK), and run using the 7500 Real-Time PCR system (Applied Biosystems, UK). Data was analysed using the 7500 Fast System Sequence Detection Software, version 1.4 (Applied Biosystems, UK).

*2.14 Isolation of alveolar macrophages for qPCR analysis*

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) +/- liposomal clodronate (100µl) i.t. day 21 and culled on day 22. Lungs were lavaged with 3 x 0.8 ml sterile Dulbecco's phosphate buffered saline (PBS) pH7.4 (D8537, Sigma, UK) containing 2mM EDTA (E5134, Sigma, UK). Lavage fluid was spun at 2000RPM for 5 minutes. Red cell lysis buffer (0.15 M $NH_4CL$ (A0171, Sigma, UK)/1mM $KHCO_3$ (P9144, Sigma, UK)/0.1mM $Na_2EDTA$ (E5134, Sigma, UK) was added to the cell pellet, incubated on ice for 5 minutes, and spun at 2000RPM for 5 minutes. Cell pellets were re-suspended in 2mls Dulbecco's modified Eagle's medium (DMEM)/F12+Glutamax (31331, Gibco, UK) and incubated at 37$^{o}$C for 1 hour. Media was gently removed and cells washed twice with PBS. RLT buffer (79216, QIAGEN, UK) plus β-mercaptoethanol (M7522, Sigma, UK) (100:1, v:v) was added, cells lysed and stored at -80$^{o}$C.

*2.15 Preparation of lung homogenates*

Lungs were thawed on ice. One protease inhibitor tablet (11836145001, Roche Applied Science, UK) was added to lysis buffer (500mM TRIS HCl (T5941, Sigma, UK)/200mM NaCl (S6150, Sigma, UK)/10mM $CaCl_2$ (C1016, Sigma, UK)) (1 tablet per 50mls). 500µl of lysis buffer/protease inhibitor solution was added to lung in a 2ml eppendorf and a steel ball bearing (69989, QIAGEN, UK) added. Lung was homogenised at 20Hz for 1 minute using a tissue lyser (85220, QIAGEN, UK). Triton X-100 (T8787, Sigma, UK) was added to make a final concentration of 0.1%. This was left on ice for 15 mins then spun at top speed (4$^{o}$C) for 5 mins. Protein extract (supernatant) was removed and stored at -80$^{o}$C.

*2.16 Arginase assay*

Arginase activity in lung homogenates was assessed, as previously described (Corraliza *et al.*, 2004), by the production of urea generated by the arginase-dependent hydrolysis of L-arginine. This is now described with modifications: lung homogenates were prepared as described earlier. 20µl sample was added to 20µl 100mM $MnCl_2$ (BDH Laboratory Supplies, Poole, UK)/50mM TRIS HCl (T5941, Sigma, UK), pH 7.4. The solution was incubated at 55$^{o}$C for 10 minutes in heat block followed by addition of 40µl 0.5M L-arginine (A5006, Sigma, UK), pH9.7. This was incubated for 1 hour at 37$^{o}$C. To this solution was added 320µl $H_2SO_4$ (BDH Laboratory Supplies, Poole, UK)/$H_3PO_4$ (P6560, Sigma, UK)/$H_2O$ (1:3:7, v:v:v) to stop the reaction. Subsequently, 20µl α-isonitrosopropiophenone (I3502, Sigma, UK) (9% solution in 100% ethanol) was added and the solution heated at 95$^{o}$C for 45-60 minutes. Aliquots were placed in 96 well plates and the absorbance read at 560nm. Arginase activity was normalised to quantity of protein as measured by BCA assay.

Standards were prepared as follows. A solution of 100mM urea in PBS was made. A twelve point standard curve was generated by serial dilutions (1:1) of the 100mM urea (U5378, Sigma, UK) solution with PBS. To each standard was added 320µl $H_2SO_4$/$H_3PO_4$/$H_2O$ (1:3:7, v:v:v) and the assay continued as for the samples, above.

*2.17 Protein quantification*

Protein quantity was assessed using the Pierce bicinchoninic acid (BCA) protein assay kit (23227, Perbio Science UK Ltd.). For lung tissue protein extracts, samples were diluted 1:6 with $H_2O$. 10µl was added to 200µl of the BCA protein assay kit working reagent (50 parts reagent A plus 1 part reagent B) in 96 well plates. Plates were incubated for 30 minutes at 37oC and absorbance read at 562nm. Standards were

supplied in the BCA protein assay kit (bovine serum albumin). Standards ranging from 0-2mg/ml (diluted in PBS) were used.

*2.18 Western Blotting*

Upper and lower gels were prepared as follows: upper gel: buffer prepared by adding 30g TRIS base (T6066, Sigma, UK) and 2g sodium dodecyl sulphate (SDS) (L3771, Sigma, UK) to 500mls $H_2O$, pH6.8. Gel stock was made by combining 50ml buffer with 32ml acrylamide (30%) (A3699, Sigma, UK) and 118mls $H_2O$. Gel was made by adding 40µl ammonium persulphate (APS) (A3678, Sigma, UK) and 20µl tetramethylethylenediamine (TEMED) (T9281, Sigma, UK) to 6mls of gel stock. Lower gel: buffer prepared by adding 90.8g TRIS base and 2g SDS to 500mls $H_2O$, pH8.8. Gel stock was made by combining 40mls buffer with 36mls glycerol (G5516, Sigma, UK), 20mls $H_2O$ and 64mls acrylamide (30%). Gel was made by adding 60µl APS and 30µl TEMED to 11mls of gel stock. 5µl of ladder was added (Precision Plus Protein Kaleidoscope Standard, 161-0375, BIO-RAD, USA). 30µl of BALF sample or 25µl of normalised lung tissue protein extract was added. Gel was run at 75V until sample within gel and then run at 120V in both upper and lower gels. Running buffer (X10) was 10g SDS, 144g Glycine (G8898, Sigma, UK) and 30.3g TRIS base in 1l $H_2O$. Blotting was performed for 1 hour (300mA). Transfer buffer was 100mls 10X transfer buffer (144g glycine and 30.3g TRIS base in 1l $H_2O$) added to 200mls methanol and 700mls $H_2O$. Blots were blocked for 1hr in 5% dry milk (Marvel) in TBS-Tween (TBST) (100mls 10X TBS(12.11g TRIS base and 90g NaCl (S3014, Sigma, UK) in 1l $H_2O$, pH7.6), 900mls $H_2O$, 0.5mls Tween-20 (P1379, Sigma, UK)). Blots were then washed twice in TBST (1 minute each in 50ml Falcon). 6mls of primary antibody was added (polyclonal goat anti-mouse TIMP1 antibody, 1:500 in 5% milk-TBST, AF980, R&D Systems, UK), incubated at $4^oC$ overnight, and washed x5 with TBST. Secondary antibody was added (rabbit anti-goat HRP, 1:2000 in 5% TBST, P0447, Dako UK Ltd, Cambridgeshire) for 1 hour. Working horseradish peroxidase (HRP) substrate was made by mixing equal volumes of luminol reagent and peroxide solution (chemiluminescent

HRP substrate, P90720, Millipore Corporation, USA), leaving for 10 minutes at room temperature, adding to blot, leaving for 5 minutes. Antibody was detected by exposing the blot to x-ray film (Amersham HyperfilmTM ECL, 28-9068-35, GE Healthcare Ltd., Buckinghamshire, UK) in a Hypercassette (Amersham Biosciences UK Ltd.) for variable exposure times. To strip blots they were incubated in stripping buffer (10mls 10X stripping buffer (7.5g TRIS base and 10g SDS in 100mls $H_2O$, pH6.7), 2mls β-mercaptoethanol (M7522, Sigma, UK) and 100mls $H_2O$, pH6.7) for 10 minutes at 65$^oC$, blots drained briefly, washed x4 (each of 5 minutes) in TBST in glass dish, washed x2 (each of 3 minutes) in TBST in 50ml falcons and blocked with 5% milk-TBST for 30 minutes. Where a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was used, primary antibody was added (monoclonal mouse anti-GAPDH antibody, 1:20,000 in 5% milk-TBST, CB1001, Calbiochem) to blot, incubated overnight at 4$^oC$, and washed x5 with TBST. Secondary antibody was added (horse anti-mouse IgG, HRP-linked antibody, 1:2000 in 5% milk-TBST, #7076, Cell Signaling Technology, USA) for 1 hour and detected as above.

*2.19 Gelatin Zymography*

Samples were subjected to electrophoresis on a 10% SDS-PAGE gel co-polymerised with 1% gelatin substrate (24350.262, BDH, UK). Following electrophoresis, gels were renatured in 2.5% Triton X-100 (T8787, Sigma, UK) for 30minutes before incubation in activity buffer (50mM TRIS base (T6066, Sigma, UK), 200mM NaCl (S3014, Sigma, UK), 5mM $CaCl_2$ (anhydrous) (C1016, Sigma, UK), 0.02% Brij-35 (B4184, Sigma, UK), pH 7.5) at 37$^oC$ for 16-24hrs. Gels were then stained with Coomassie Brilliant Blue R250 (27816, Sigma, UK) before destaining in 40% methanol (C-M400017-X, Fisher Scientific, UK), 10% acetic acid (A6283, Sigma, UK). Gelatinolytic activity was detected as destained bands against a background of Coomassie brilliant blue stained gelatin and the molecular weight correlated against the included protein ladder (Benchmark prestained protein ladder, 10748-010, Invitrogen, UK).

*2.20 Preparation and stimulation of bone marrow derived macrophages*

In preparing cells for intra-tracheal "add-back" experiments, primary murine bone marrow-derived macrophages (BMDMs) were prepared from C57Bl/6 mice as follows: bones were removed from mice and placed in GIBCO$^{TM}$ Hank's balanced salt solution (14170120, Invitrogen, UK). Bones were stripped of flesh and ends removed. Bone marrow was obtained using standard sterile techniques from femurs and tibias by flushing through with 10mls of media (Dulbecco's modified Eagle's medium (DMEM)/F-12 (with glutamax) (31331, Invitrogen, UK) with 10% heat inactivated fetal bovine serum (FBS) (S1900-500, Biosera, Ringmer, UK) 20% L929 cell conditioned medium and 1% penicillin-streptomycin (P11-010, PAA Laboratories Ltd., UK) per bone. Cells were matured for 7 days in sterile Teflon pots (Roland Vetter Laborbedarf OHG, Germany). 25% of the media was replaced on alternate days. If stimulation was required, on the 6th night cells were pelleted by centrifugation at 300g for 5 minutes, washed twice with PBS, and serum starved overnight (in DMEM/F-12 with 1% penicillin-streptomycin). Cells were then spun down again at 300g for 5 minutes and re-suspended in stimulating media; for alternative activation they were re-suspended in DMEM/F-12 with 1% penicillin-streptomycin and stimulated with recombinant mouse interleukin-4 (IL-4) (404-ML/CF, R&D Systems, UK) at a concentration of 10ng/ml for 24 hours. For classical activation they were re-suspended in DMEM/F-12 with 1% penicillin-streptomycin and stimulated with recombinant interferon-γ (IFN-γ) (485-MI, R&D Systems, UK) at a concentration of 20ng/ml and lipopolysaccharide (LPS) (L4516, Sigma, UK) at a concentration of 50ng/ml for 24 hours. Cells were pelleted by centrifugation at 300g for 5 minutes, washed twice with PBS, re-suspended in PBS at a concentration of $1x10^6$ cells per 50µl, and immediately administered intra-tracheally.

*2.21 Flow cytometry*

BAL

Mice were culled as described previously. Lungs were lavaged with 4x0.8mls of ice cold, sterile PBS supplemented with 2mM EDTA. Cells were pelleted by spinning at 2000RPM for 5 minutes then re-suspended in 0.5mls red cell lysis buffer (0.15 M $NH_4CL$ (A0171, Sigma, UK)/1mM $KHCO_3$ (P9144, Sigma, UK)/0.1mM $Na_2EDTA$ (E5134, Sigma, UK). Samples were lysed on ice for 5 minutes, spun at 2000RPM for 5 minutes, then re-suspended in FACS buffer (PBS/0.5% bovine serum albumin (A7906, Sigma, UK)). Cytospins were created as previously described. Fc block™ (rat anti-mouse CD16/CD32, 1:500, 553142, BD Europe) was added to samples and incubated at 4°C for 15 minutes. 1ml of FACS buffer was added, samples spun at 2000RPM for 5 minutes and re-suspended in FACS buffer. Antibodies were added (PE anti-mouse I-Ab (MHC II), 1:200, 553104, BD Europe; PERCP-Cy5.5 anti-mouse CD11b, 1:200, 45-0112-80, eBioscience Ltd., UK; APC anti-mouse CD11c, 1:200, 550261, BD Europe; FITC rat IgG2aκ isotype, 553457, BD Europe; PERCP-Cy5.5 rat IgG2bκ isotype, 550764, BD Europe; APC hamster IgG isotype, #0300A/HM05, Caltag Laboratories, UK) and stained on ice for 30 minutes. 1ml FACS buffer was added, samples spun at 300g for 5 minutes, re-suspended in 200µl FACS buffer and analysed using a BD FACS Calibur Flow Cytometer: 4-Color. Data was analysed using FlowJo software, version 7.2.4 (Tree Star Inc., USA).

Lung

Mice were culled as described previously. Lungs were removed and placed in GIBCO™ Hank's balanced salt solution (14170120, Invitrogen, UK). Lungs were placed in 2ml digestion mixture (collagenase D (#11088866001, Roche Applied Sciences, UK) in FACS buffer, 4mg/ml), minced into a "slurry" using scissors, and digested at 37°C for 1 hour. Cells were released from tissue by vigorous pippetting, spun at 300g for 15 minutes and washed with FACS buffer. After a further spin at 300g for 5 minutes, 2mls red cell lysis buffer (0.15 M $NH_4CL$ (A0171, Sigma, UK)/1mM

$KHCO_3$ (P9144, Sigma, UK)/0.1mM $Na_2EDTA$ (E5134, Sigma, UK) was added and lung mix lysed for 5 minutes on ice. Samples were topped up with FACS buffer, spun at 300g for 5 minutes and re-suspended in FACS buffer before pouring through a 40µm cell strainer (352340, BD Biosciences, UK). This single cell suspension was spun at 300g for 5 minutes, and re-suspended in FACS buffer. An aliquot was used for cell counting, using nucleocassettes (941-0002, Chemometec, Denmark) and a nucleocounter (SCC-100, Chemometec, Denmark). Fc block$^{TM}$ (rat anti-mouse CD16/CD32, 1:500, 553142, BD Europe) was added to the lung suspension for 15 minutes at 4$^{o}$C. Samples were topped up with FACS buffer and spun at 300g for 5 minutes. FACS buffer was added to produce a final concentration of $1x10^7$ cells per ml. Antibodies were added (FITC anti-mouse Ly6G, 1:100, 127605, Biolegend, USA; PERCP-Cy5.5 anti-mouse CD11b, 1:200, 45-0112-80, eBioscience Ltd., UK; APC anti-mouse CD11c, 1:200, 550261, BD Europe; FITC rat IgG2a isotype, 11-4321-81, BD Europe; PERCP-Cy5.5 rat IgG2bκ isotype, 550764, BD Europe; APC hamster IgG isotype, #0300A/HM05, Caltag Laboratories, UK) to 200µl aliquots and stained on ice for 30 minutes. 1ml FACS buffer was added, samples spun at 300g for 5 minutes, re-suspended in 200µl FACS buffer and analysed as above.

Blood

Mice were anaesthetised with a lethal dose of pentobarbital sodium as previously described. Blood was removed from the inferior vena cava and immediately added to 3.9% citrate (1:1). Aliquots of 60µl were used for subsequent analysis. Antibodies were added (FITC anti-mouse Ly6G, 1:120, 127605, Biolegend, USA; PE anti-mouse CD115, 1:120, 12-1152-82, eBioscience Ltd., UK; PERCP-Cy5.5 anti-mouse CD11b, 1:240, 45-0112-80, eBioscience Ltd., UK; Alexa Fluor 647 anti-mouse 7/4, 1:240, MCA771A647, AbD Serotec, UK; FITC rat IgG2a isotype, 11-4321-81, BD Europe; PE rat IgG2b isotype, 12-4321-81, eBioscience Ltd., UK; PERCP-Cy5.5 rat IgG2bκ isotype, 550764, BD Europe; Alexa Fluor 647 rat IgG2a isotype, MCA1124A647, AbD Serotec, UK) and stained on ice for 30 minutes. Red blood cells were lysed by adding 0.5mls of 1x FACS

lysing solution (349202, BD Europe), vortexing the solution and incubating for 10 minutes at room temperature in the dark. FACS buffer was added, samples spun at 300g for 5 minutes, washed with FACS buffer and re-suspended in 200µl FACS buffer and analysed as above.

*2.22 Flow sorting*

In order to produce a single cell suspension for the "add-in" experiments, bone marrow was retrieved as described previously. Cells were then counted using a Nucleocounter as described previously. Fc block™ (rat anti-mouse CD16/CD32, 1:500, 553142, BD Europe) was added and incubated for 15 minutes at 4°C. Cells were spun at 300g for 5 minutes and re-suspended in GIBCO™ Hank's balanced salt solution (14170120, Invitrogen, UK) at a concentration of $1x10^7$ cells per ml. Antibodies were added (FITC anti-mouse Ly6G, 1:100, 127605, Biolegend, USA; PE anti-mouse CD115, 1:100, 12-1152-82, eBioscience Ltd., UK; PERCP-Cy5.5 anti-mouse Ly6C, 1:100, 45-5932-82, eBioscience Ltd., UK; APC anti-mouse CD11b, 1:100, 553312, BD Europe) and stained for 30 minutes on ice. Cells were spun at 300g for 5 minutes and re-suspended in GIBCO™ Hank's balanced salt solution/1% FBS (S1900-500, Biosera, Ringmer, UK) at a concentration of $4x10^7$ cells/ml. Cells were sorted as described in results into a solution of RPMI 1640 (G0029, 3010, PAA Laboratories Ltd., UK) / 10% FBS. Cells were counted, re-suspended in RPMI 1640 at a concentration of $5x10^6$ cells/ml, and 200µl ($1x10^6$cells) injected into the tail vein of mice. All methods performed were in sterile conditions. Furthermore, the BD FACS Aria II (Special order system) was specifically designed with sterility in mind, and has several features that minimise the occurrence of contamination and facilitate the cleaning and decontamination of the fluidics system, ensuring optimal aseptic conditions as far as possible..

*2.23 Statistical analysis*

Paired data columns were evaluated using a two-tailed Student's t test with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). As bleomycin is the positive control throughout, and all comparisons are made directly with that, then the Student's t-test is believed to be the preferred choice of statistical analysis. Where multiple groups are involved, the relation of each to another is not believed to be stastically relevant; only the relationship to the positive control, and for this reason the Student's t-test was used here also. Data were tested for normality and variance. Fibrosis score is presented as box and whiskers, inter-quartile range, minimum to maximum. Significance of the difference between medians was assessed using a Mann Whitney test with GraphPad Prism. A P value less than 0.05 was considered significant.

*2.24 Human tissue samples*.

Written informed consent was obtained from all subjects. The study was approved by the Lothian Research Ethics Committee. Bronchoalveolar lavage was performed as part of ongoing clinical research studies. All cases were discussed at the Lothian clinicopathoradiological meeting. Patients were defined as having IPF by ATS/ERS non-biopsy criteria.

# CHAPTER 3

## ANIMAL MODELS OF PULMONARY FIBROSIS

### *3.1 Abstract*

Many attempts have been made over the years to produce the optimal model of pulmonary fibrosis. The widely accepted gold standard is the bleomycin model of pulmonary fibrosis. Bleomycin is classically given by intra-tracheal route, although may be given intra-peritoneally or intra-venously. A criticism of the intra-tracheal route is that it produces a bronchiolocentric pattern of lung fibrosis, following a significant period of lung inflammation, in contrast with the peripheral pattern of injury and "pure" fibrosis deemed to be present in the archetypal human form of pulmonary fibrosis, IPF. A second model that has been established is the adenoviral transforming growth factor-β (AdTGFβ) model. When AdTGFβ is given by intra-tracheal route it is believed to produce a more peripheral fibrosis, with minimal inflammation, and as such many researchers believe is more representative of the human form of pulmonary fibrosis. In investigating the role of monocytes and macrophages in pulmonary fibrosis, I sought to use these two models to ensure that any results were not biased to a particular model, but were representative of the effects of these cell types in pulmonary fibrosis *per se*.

*3.2 Introduction*

Bleomycin is the best characterised model of pulmonary fibrosis. As mentioned previously, it was first given to animals in experimental form by Robert Fleischman and colleagues (Fleischman *et al*., 1971) and was subsequently given to mice intra-peritoneally by Adamson and Bowden (Adamson & Bowden 1974) and intra-tracheally by Raisfeld and colleagues (Raisfeld *et al*., 1982). In the early 1980s most of the bleomycin studies were performed in rabbits, hamsters and rats. It is well known that the response to bleomycin is variable dependent on factors such as age, sex, and strain of mice, C57Bl/6 mice being more susceptible to bleomycin-induced fibrosis than Balb/c mice (Schrier *et al*., 1983). Furthermore, it is known that bleomycin has a variable activity range (Stefanou & Siderov 2001), and as such the source from which the bleomycin is obtained will be a factor in the model of bleomycin-induced pulmonary fibrosis that is produced. It is therefore imperative that any research performed using the bleomycin model is preceded by an optimisation of the model in that specific research facility. As such I optimised the bleomycin model as will subsequently be described.

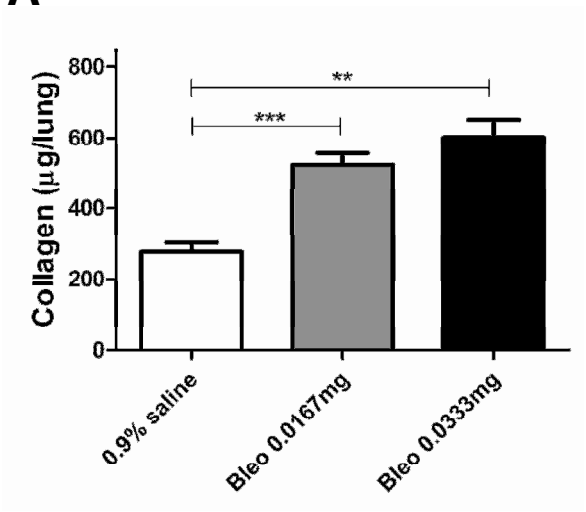
In order to ensure that any findings demonstrated in the bleomycin model were attributable to a monocyte/macrophage effect on pulmonary fibrosis, rather than a monocyte/macrophage effect on bleomycin lung injury, I used a second model of pulmonary fibrosis, that of the AdTGFβ model. This model was developed by Patricia Sime in Jack Gauldie's laboratory and as described earlier uses a replication deficient adenoviral vector that expresses biologically active $TGF\beta_1$ (Sime *et al*., 1997). Given intra-tracheally it causes lung fibrosis with minimal inflammation in both rats (Sime *et al*., 1997) and mice (Warshamana *et al*., 2002) and in a pattern more resemblant of IPF. As a result it can be used as an adjunct to study the effects of monocytes and macrophages in lung fibrosis.

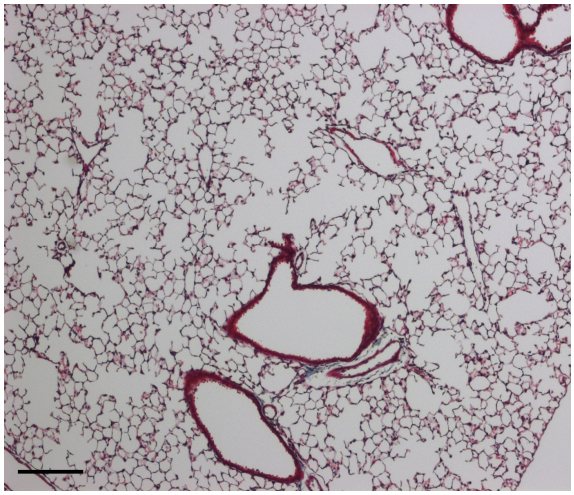
*3.3 Results*

**Bleomycin-induced pulmonary fibrosis is affected by dose given**

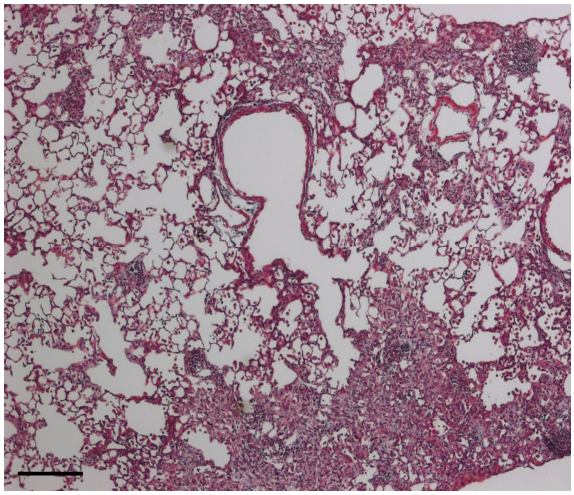
To determine an optimal dose of bleomycin to be used in subsequent experiments, two doses of bleomycin were chosen – 0.0167mg and 0.033mg (by weight) (Figure 3.1A). These are approximately equivalent to 0.025mg and 0.05mg (by potency), and thus are approximately equivalent to 0.025U and 0.05U, respectively. (IU is equivalent to 1mg by potency). As described in numerous publications a dose of approximately 0.025U is a "standard" dose and 0.05U a "high" dose. Bleomycin was given intra-tracheally (i.t.). 0.9% saline was given i.t. as a control. Mice were culled on day 18. As shown in Figure 3.1A, there is an increase in pulmonary fibrosis as measured by Sircol assay and confirmed by Masson's trichrome staining of lung sections (Figure 3.1, B-D).when the larger dose of 0.033mg is given. However this effect was not statistically significant and may simply reflect the small numbers used.

# Figure 3.1

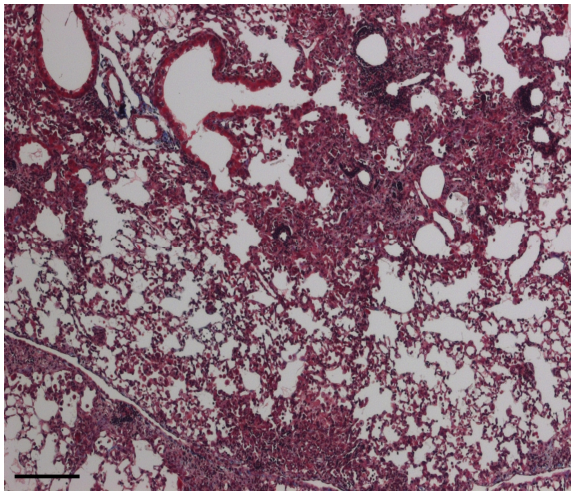



0.9% saline




Bleomycin 0.0167mg




Bleomycin 0.033mg

Figure 3.1

**Bleomycin-induced pulmonary fibrosis is affected by dose given**

Female C57Bl/6 mice were given 0.9% saline, 0.0167mg or 0.033mg of bleomycin intra-tracheally (i.t.) (50µl) and culled on day 18. Fibrosis was quantified by Sircol assay (A) or visualised by Masson's trichrome staining (B)-(D). (A) Bleomycin induces pulmonary fibrosis which is affected by dose given: 0.9% saline vs. bleomycin 0.0167mg, ***p=0.0009; 0.9% saline vs. bleomycin 0.033mg, **p=0.0011. (B)-(D) Representative images of lung sections stained by Masson's trichrome where the substances given i.t. were: (B) 0.9% saline, (C) bleomycin 0.0167mg, and (D) bleomycin 0.033mg. n=4-5 per group. Data are presented as mean +/- SEM. Bar 200µm.

**Bleomycin-induced pulmonary fibrosis is reversible**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. and culled on days 3, 7, 14, 21, 28, 56 and 136. 0.9% saline was given i.t. as a control and mice culled on day 3. As demonstrated in Figure 3.2A, pulmonary fibrosis as measured by Sircol assay peaks around 28 days. By 56 days fibrosis has significantly reduced, with a further reduction by day 136. This increase in fibrosis to day 28 is confirmed by Masson's trichrome staining of lung sections (Figure 3.2, B-G) with reversibility seen thereafter (Figure 3.2, H-I).

# Figure 3.2

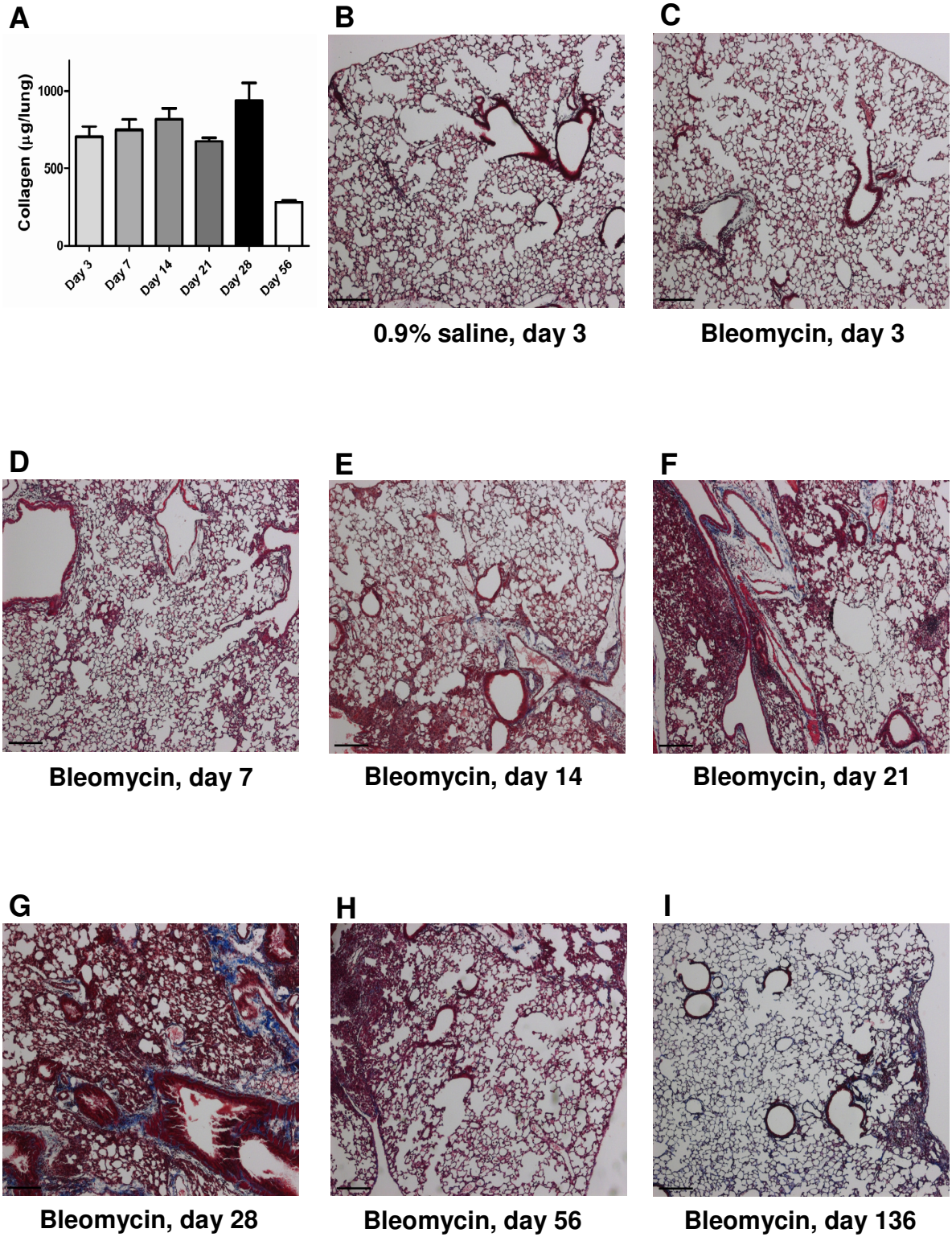

A
B
C
Collagen (µg/lung)
1000
500
0
Day 3
Day 7
Day 14
Day 21
Day 28
Day 56
0.9% saline, day 3
Bleomycin, day 3
D
E
F
Bleomycin, day 7
Bleomycin, day 14
Bleomycin, day 21
G
H
I
Bleomycin, day 28
Bleomycin, day 56
Bleomycin, day 136

Figure 3.2

**Bleomycin-induced pulmonary fibrosis is reversible**

Female C57Bl/6 mice were given 0.033mg of bleomycin i.t. (50µl) and culled on day 3, 7, 14, 21, 28, 56, 136. 0.9% saline was given i.t. as a control and mice culled on day 3. Fibrosis was measured by Sircol assay (A) or demonstrated by Masson's trichrome staining (B)-(I). (A) Bleomycin induces pulmonary fibrosis which peaks around 28 days and exhibits reversibility thereafter. (B)-(I) Representative images of lung sections stained by Masson's trichrome: (B) 0.9% saline, (C) bleomycin, day 3, (D) bleomycin, day 7, (E) bleomycin, day 14, (F) bleomycin, day 21, (G) bleomycin, day 28, *(H) bleomycin, day 56, (I) bleomycin, day 136. n=3-4 per group, except *n=2. Data are presented as mean +/- SEM. Bar 200µm.

**Macrophages increase in number during bleomycin-induced pulmonary fibrosis**

Female C57Bl/6 mice were given 0.033mg of bleomycin i.t. (50µl) and culled on day 3, 7, 14, 21, 25 and 28. 0.9% saline was given i.t. as a control and mice culled on day 14. Sections were stained for the presence of the F4/80 antigen. The F4/80 antigen is a macrophage restricted marker (McKnight & Gordon 1998) and therefore is an ideal target for immunohistochemical localisation of tissue macrophages. Using immunohistochemistry I demonstrate that macrophage numbers appear to increase during bleomycin-induced pulmonary fibrosis, as shown in Figure 3.3, A-F, with a possible peak between 21 and 28 days. This was not formally quantified, but the appearances were consistent on multiple sections analysed. Macrophages are present in control mice (Figure 3.3G), but their numbers are markedly reduced compared to bleomycin-treated animals. There is no staining when an isotype control was used instead of primary antibody (Figure 3H) or when a no primary control was performed (Figure 3.3I). With all antibodies, the question has to be asked whether there is the possibility that macrophage subtypes change (during disease progression), and that the F4/80 staining may simply reflect F4/80-positive macrophage subtypes at particular phases of pulmonary fibrosis. However, there is no evidence to date that F4/80-positive macrophages in tissue reflect a particular macrophage subtype.

# Figure 3.3

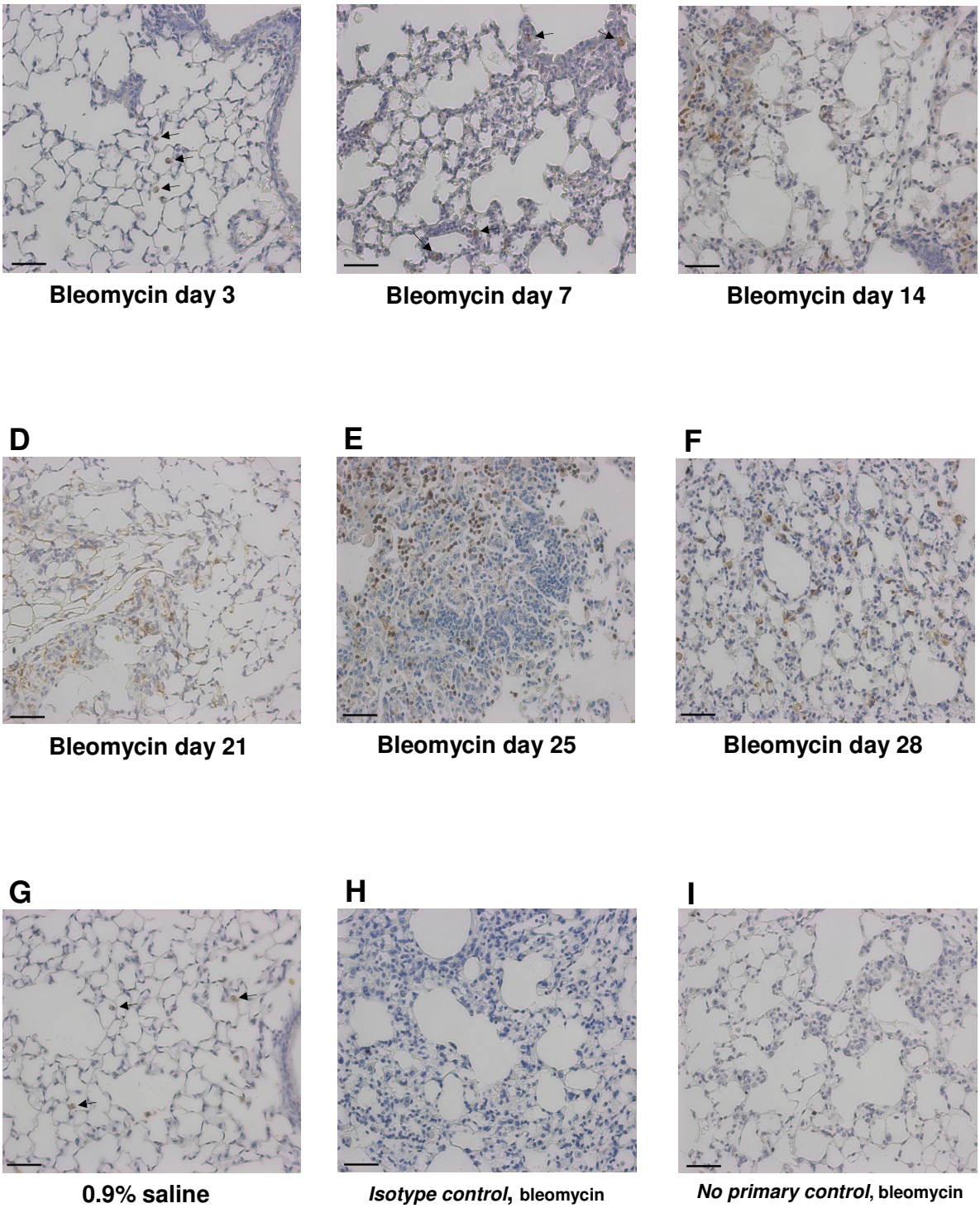

A
B
C
Bleomycin day 3
Bleomycin day 7
Bleomycin day 14
D
E
F
Bleomycin day 21
Bleomycin day 25
Bleomycin day 28
G
H
I
0.9% saline
Isotype control, bleomycin
No primary control, bleomycin

Figure 3.3

**Macrophages increase in number during bleomycin-induced pulmonary fibrosis**

Female C57Bl/6 mice were given 0.033mg of bleomycin i.t. (50µl) and culled on day 3, 7, 14, 21, 25 and 28. 0.9% saline was given i.t. as a control and mice culled on day 14. Immunohistochemistry was performed for the F4/80 antigen (A)-(F). An isotype control was given instead of primary antibody (G), and a no primary control was also performed (H). (A) Mice given bleomycin and culled on day 3 show minimal staining for the F4/80 antigen. (B) Mice given bleomycin and culled on day 7 show minimal staining for F4/80. (C) Mice given bleomycin and culled on day 14 show an increase in staining for F4/80. (D) Mice given bleomycin and culled on day 21 show a marked increase in F4/80 staining. (E) Mice given bleomycin and culled on day 25 show a persistence of a marked increase in F4/80 staining. (F) Mice given bleomycin and culled on day 28 show a persistence of a marked increase in F4/80 staining. (G) Mice given 0.9% saline and culled on day 14 show some minimal staining for F4/80. (H) Mice given bleomycin and culled on day 25 show no staining when an isotype control antibody was used instead of the F4/80 primary antibody. (I) Mice given bleomycin and culled on day 21 show no staining when secondary, but no primary, antibody was given. Arrow: F4/80 stained macrophages. Bar 50µm.

**Myofibroblasts increase in number during bleomycin-induced pulmonary fibrosis**

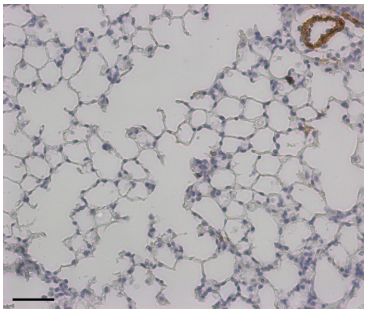
Female C57Bl/6 mice were given 0.033mg of bleomycin i.t. (50µl) and culled on day 3, 7, 14, 21, and 28. 0.9% saline was given i.t. as a control and mice culled on day 14. Sections were stained for the presence of the α-smooth muscle actin (α-SMA) antigen. The α-SMA antigen is a marker of myofibroblasts, a term originally coined by Gabbiani and colleagues (Gabbiani *et al*., 1971). Myofibroblasts are believed to contribute significantly to collagen deposition (Zhang *et al*., 1994) and play a major role in the pathogenesis of pulmonary fibrosis (Zhang *et al*., 1994; Gharaee-Kermani *et al*., 2009). Using immunohistochemistry I have established that myofibroblast numbers increase in my model of bleomycin-induced pulmonary fibrosis, as shown in Figure 3.4, A-E, with an apparent peak between 14 and 28 days. The apparent peak is not an absolute description. The difficulty with a non solid organ such as the lung is that in any one lung histological section, staining for myofibroblasts can be seen to be positive, but as the shape of myofibroblasts is such that they have long projections, any positive staining is not quantifiable in terms of cell numbers. An alternative approach is to quantify the percentage of positive staining, which is possible in the liver and kidney. However, as the lung consists of so much air, then this method of quantification is also not possible. Therefore, while the apparent peak of myofibroblast staining is between 14 and 28 days, it is not possible to be absolute sure that this is true. However, in all lung sections analysed it is clear that this increase in staining between 14 to 28 days is consistent, and is probably therefore a true effect. In control mice myofibroblasts are only present in vessels (Figure 3.4F). There is no staining within the lung interstitium or in vessels when a no primary control was performed (Figure 3.4G).

One question that can be asked is whether α-SMA defines all myofibroblasts, and therefore whether α-SMA staining is sensitive in the detection of myofibroblasts. While it is likely that there is considerable fibroblast heterogeneity (Phan 2008), it is well established that α-SMA-positive fibroblasts are myofibroblasts, and these are the predominant source of type I collagen and fibrogenic cytokines in the lung, as well as

exerting mechanical effects on fibrotic tissue (Zhang *et al*., 1994, Phan 2002). Whether further subtypes exist or not has yet to be determined.

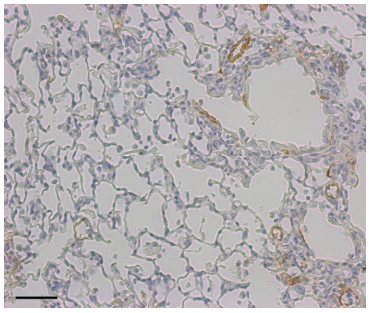
# Figure 3.4

A



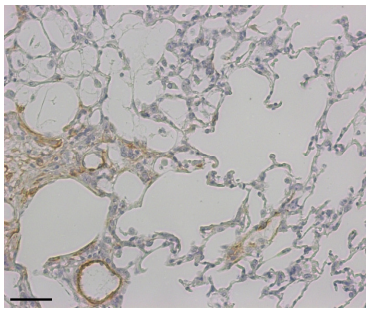
Bleomycin day 3

B



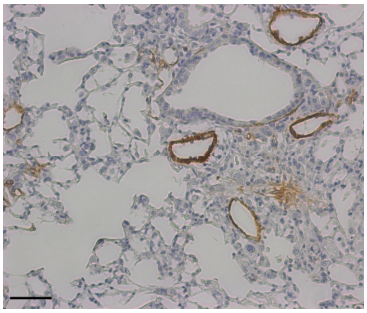
Bleomycin day 7

C



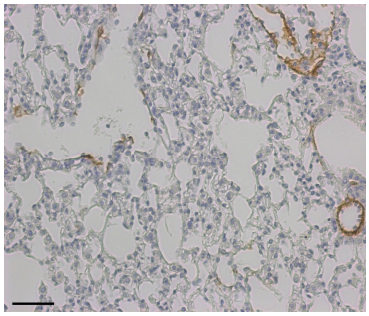
Bleomycin day 14

D



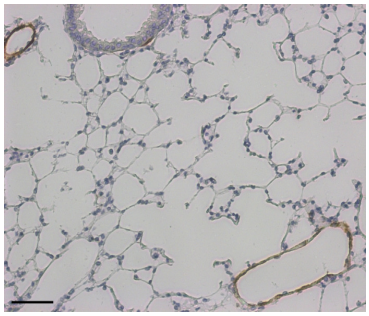
Bleomycin day 21

E



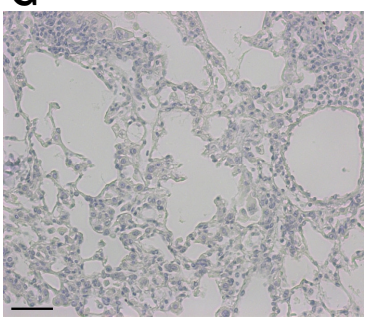
Bleomycin day 28

F



0.9% saline

G



***No Primary control*, bleomycin**

Figure 3.4

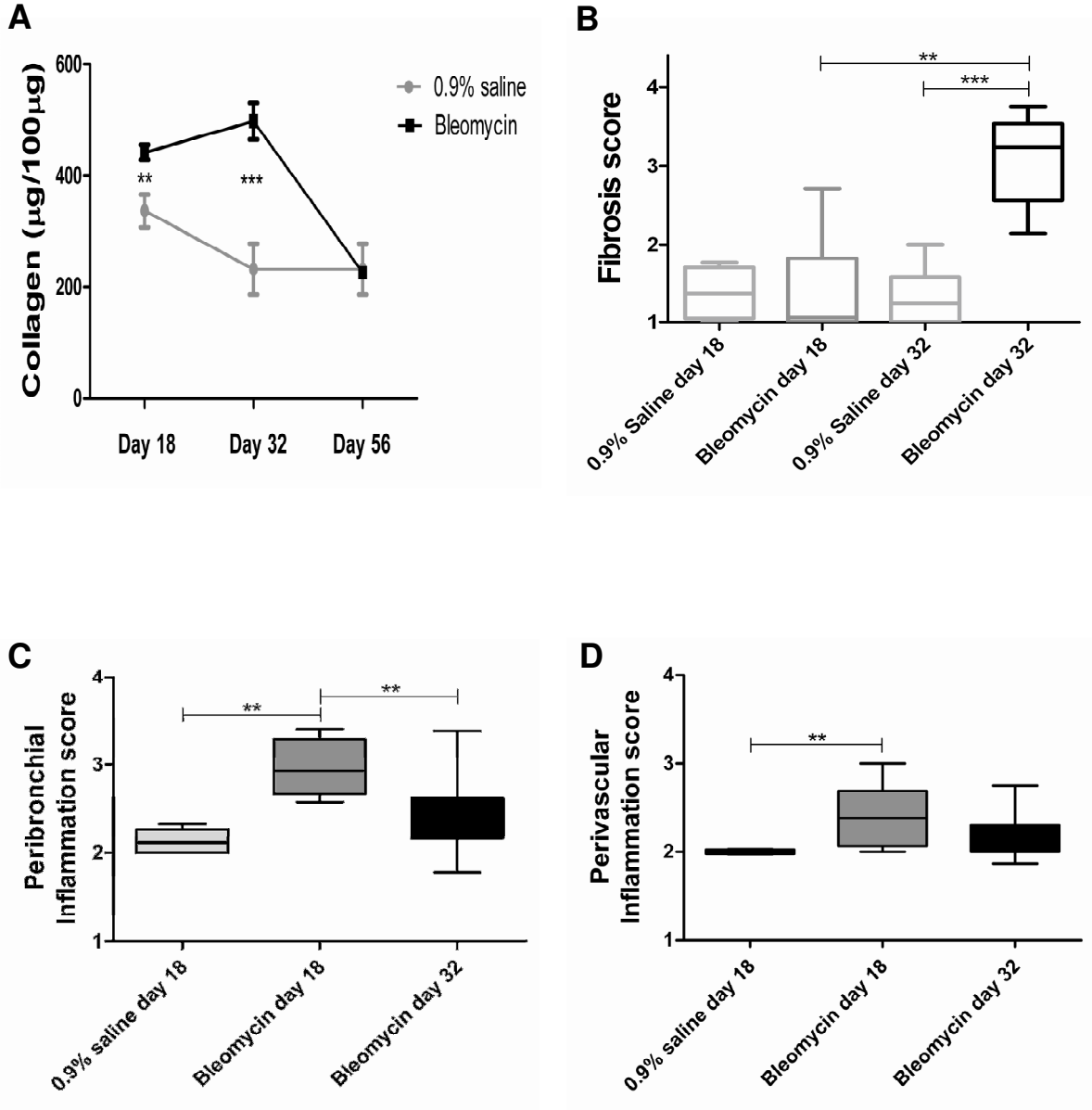
**Myofibroblasts increase in number during bleomycin-induced pulmonary fibrosis**

Female C57Bl/6 mice were given 0.033mg of bleomycin i.t. (50μl) and culled on day 3, 7, 14, 21 and 28. 0.9% saline was given i.t. as a control and mice culled on day 14. Immunohistochemistry was performed for the α-SMA antigen (A)-(F). A no primary control was also performed (G). (A) Mice given bleomycin and culled on day 3 show no staining within the lung interstitium, but appropriate staining within vessels. (B) Mice given bleomycin and culled on day 7 show some staining within the lung interstitium. (C) Mice given bleomycin and culled on day 14 show an increase in staining within the lung interstitium. (D) Mice given bleomycin and culled on day 21 show an increase in staining within the lung interstitium. (E) Mice given bleomycin and culled on day 28 show an increase in staining within the lung interstitium. (F) Mice given 0.9% saline and culled on day 14 no staining within the lung interstitium but appropriate staining within vessels. (G) Mice given bleomycin and culled on day 28 show no staining when secondary, but no primary, antibody was given. Bar 50μm.

**Stages of bleomycin-induced pulmonary fibrosis**

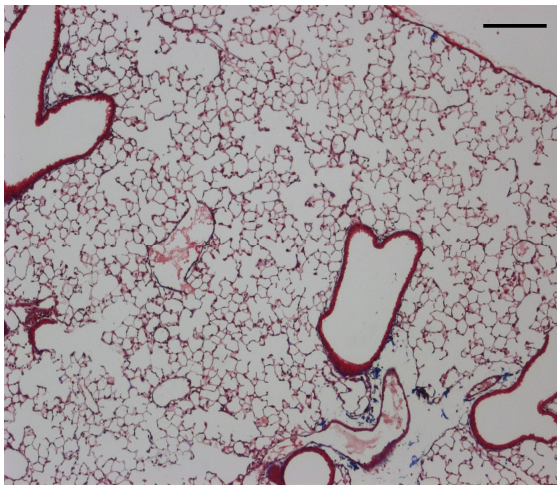
Following on from the previous results I sought to consolidate my earlier findings of the stages of pulmonary fibrosis using higher 'n' numbers, and further defining the model in some more detail. Female C57Bl/6 mice, 10-14 weeks old and weight matched, were given 0.033mg bleomycin i.t. (50μl) and culled on days 18, 32 and 56. Fibrosis was quantified by Sircol assay on days 18, 32 and 56 and corroborated with histopathological fibrosis score on days 18 and 32. Peri-bronchial and peri-vascular inflammation scores were performed on days 18 and 32. As can be seen in my model, administration of 0.033mg bleomycin i.t. to female C57Bl/6 mice reliably causes acute inflammation, followed by early fibrosis at 18 days (Figure 3.5, A, B and F) and peak fibrosis at 32 days (Figure 3.5, A, B and G). Peri-vascular and peri-bronchial inflammation increase until day 18 and decrease thereafter (Figure 3.5, C and D). Furthermore I demonstrate that fibrosis is reversible after 32 days, with a significant reduction in lung fibrosis occurring by day 56 as measured by Sircol assay (Figure 3.5A) and confirmed by morphologic assessment (Figure 3.5H).

# Figure 3.5.1


A
Collagen (µg/100µg)
600
400
200
0
0.9% saline
Bleomycin
**
***
Day 18
Day 32
Day 56
B
Fibrosis score
4
3
2
1
**
***
0.9% Saline day 18
Bleomycin day 18
0.9% Saline day 32
Bleomycin day 32
C
Peribronchial Inflammation score
4
3
2
1
**
**
0.9% saline day 18
Bleomycin day 18
Bleomycin day 32
D
Perivascular Inflammation score
4
3
2
1
**
0.9% saline day 18
Bleomycin day 18
Bleomycin day 32

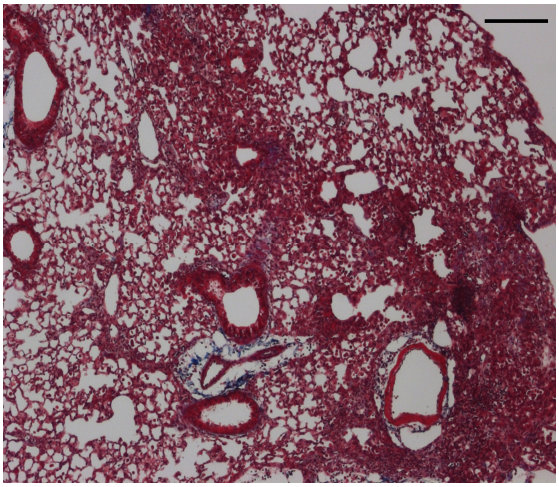
# Figure 3.5.2

E



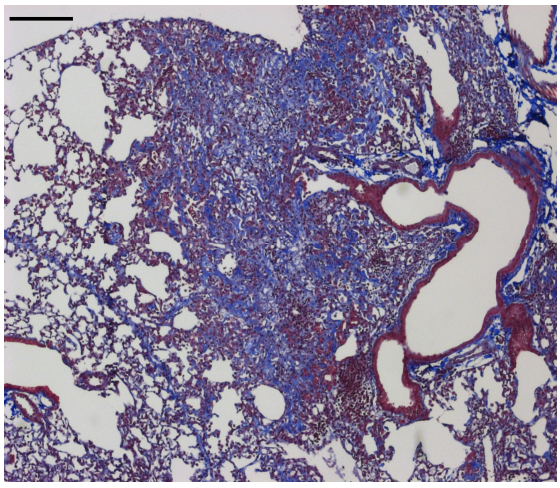
0.9% saline, day 32

F



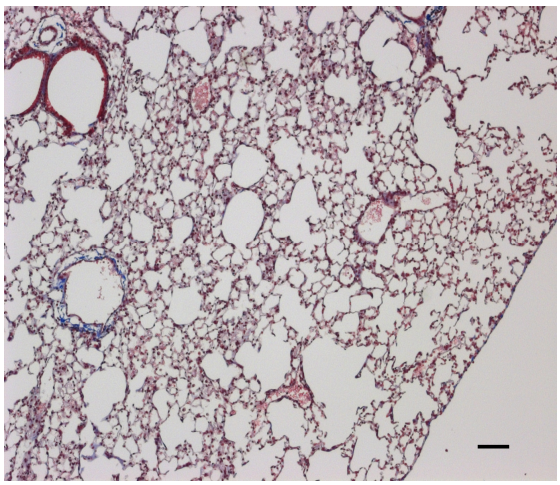
Bleomycin, day 18

G



Bleomycin, day 32

H



Bleomycin, day 56

Figure 3.5

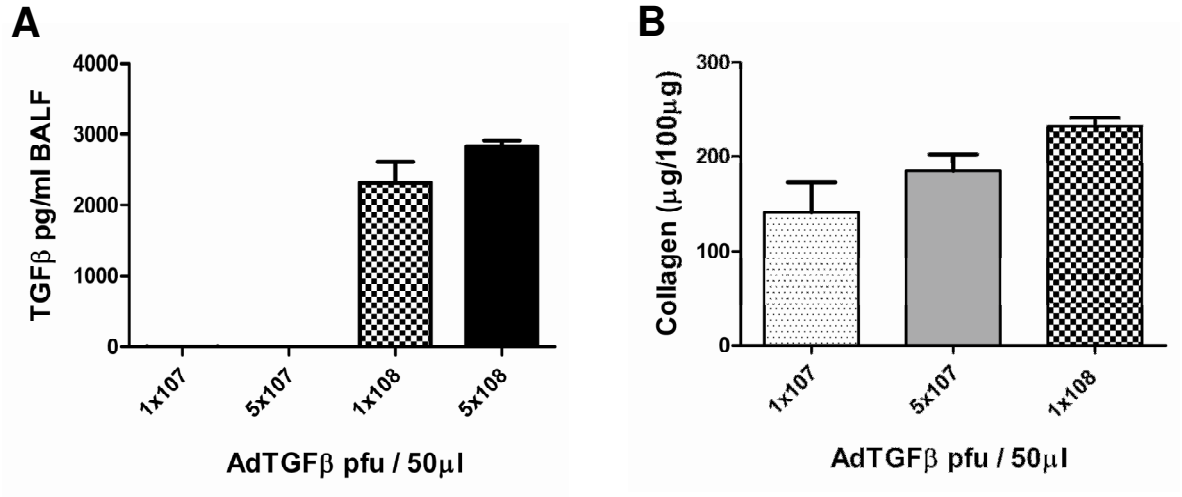
**Stages of bleomycin-induced pulmonary fibrosis**

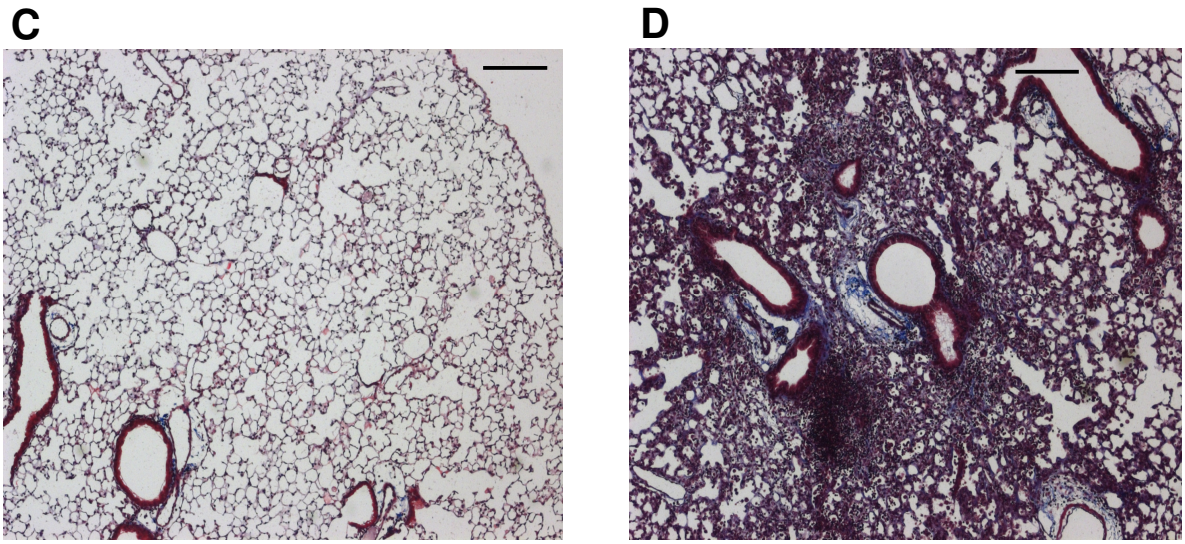
Female C57Bl/6 mice were given 0.033mg bleomycin intra-tracheally (i.t., 50μl) and culled on days 18, 32 and 56. 0.9% saline was given as a control. Fibrosis was measured by Sircol assay (A) or semi-quantitative fibrosis score (B). Inflammation was assessed by semi-quantitative peri-bronchial (C) and peri-vascular (D) scores. (A) Mice were given 0.9% saline or bleomycin by i.t. instillation, culled during early lung fibrosis (day 18), progressive fibrosis (day 32), fibrosis resolution (day 56), and lung collagen assessed. Significant differences in lung collagen were found between 0.9% saline-treated and bleomycin-treated groups at both 18 days (**, p=0.0051) and 32 days (***, p=0.0008). By 56 days collagen levels had returned to near 0.9% saline levels. (B) Mice given bleomycin were culled at 18 or 32 days and fibrosis score assessed. Mice given 0.9% saline were culled at 18 or 32 days and fibrosis score assessed. Significant differences in fibrosis score were found between mice culled at 32 days (0.9% saline vs. bleomycin, ***, p=0.0002) and between mice given bleomycin and culled at day 18 or day 32 (**, p=0.0011). (C) Mice were given 0.9% saline and culled at 18 days, or bleomycin and culled at 18 and 32 days. Peri-bronchial inflammation score was assessed. Significant differences in score were found between mice culled at 18 days (0.9% saline vs. bleomycin, **, p=0.0024) and between mice given bleomycin and culled at day 18 or day 32 day (**, p=0.0073). (D) Mice were given 0.9% saline and culled at 18 days, or bleomycin and culled at 18 and 32 days. Peri-vascular inflammation score was assessed. Significant differences in score were found between mice culled at 18 days (0.9% saline vs. bleomycin, **, p=0.0047). There was a trend to reduction in score between mice given bleomycin and culled at day 18 or day 32 day. (E) Masson's trichrome stain, 0.9% saline day 32. (F) Masson's trichrome stain, bleomycin day 18. (G) Masson's trichrome stain, bleomycin day 32. (H) Masson's trichrome stain, bleomycin day 56. n=4-8 for each group. Data are presented as (A) mean +/- SEM, and (B)-(D) Box and whiskers (Median, Inter-quartile range, minimum to maximum). Bar 200μm (E)-(G), 100μm (H).

**Adenoviral transforming growth factor-β (AdTGFβ) induces pulmonary fibrosis**

Administration of AdTGFβ by i.t. route has been shown to induce prominent lung fibrosis (Sime *et al*., 1997; Warshamana *et al*., 2002). Female C57Bl/6 mice, 10-14 weeks old and weight matched, were given a range of doses of AdTGFβ i.t. and culled on day 5 to assess TGFβ levels in the bronchoalveolar lavage fluid (BALF), and on day 14 to assess pulmonary fibrosis. In my hands I have established that at least $1x10^8$ pfu/50μl of AdTGFβ administered by i.t. route were required to ensure that TGFβ was detectable in bronchoalveolar lavage fluid (BALF) after 5 days as measured by ELISA (Figure 3.6A). Fibrosis measured at 14 days by Sircol assay was dose dependent (Figure 3.6B). At quantities of AdTGFβ of $5x10^8$ pfu/50μl mice became unwell and had to be culled early. Administration of empty viral vector (AdDL70-3) did not induce lung fibrosis as seen on lung sections stained by Masson's trichrome (Figure 3.6C) or as measured by Sircol assay (Figure 3.6A). Administration of AdTGFβ causes significant fibrosis as can be seen on lung sections stained by Masson's trichrome (Figure 3.6D).

# Figure 3.6




AdDL70-3, day 14

AdTGFβ, day 14

Figure 3.6

**Adenoviral transforming growth factor-β (AdTGFβ) induces pulmonary fibrosis**

Female C57Bl/6 mice were given adenoviral-TGFβ (AdTGFβ) intra-tracheally (i.t., 50μl). AdDL70-3, empty viral vector, was given as a control. (A) Administration of AdTGFβ results in production of TGFβ, as measured in bronchoalveolar lavage fluid (BALF), and is dose-dependent. (B) AdTGFβ induces pulmonary fibrosis as measured by Sircol assay and is dose-dependent. (C) Masson's trichrome staining 14 days following administration of AdDL70-3 ($1x10^8$ pfu/50μl). (D) Masson's trichrome staining 14 days following administration of AdTGFβ ($1x10^8$ pfu/50μl). (A)-(B) n=3 for each group. (C)-(D) n=4-5. Bar 200μm.

*3.4 Discussion*

I have shown that I have developed a model of pulmonary fibrosis induced by administration of bleomycin intra-tracheally. I have shown that 0.033mg (~0.05U) of bleomycin may be administered safely to induce lung injury characterised by very early inflammation, early fibrosis at 18 days, and peak fibrosis around 28-32 days. Furthermore I have shown that during injury there is an increase in macrophage numbers with a peak around 21-28 days, and a concurrent increase in myofibroblast numbers with a peak between 14 and 28 days. By 56 days, pulmonary fibrosis has significantly resolved, and is absent by 136 days.

As has been mentioned previously, although bleomycin-induced lung fibrosis is the best characterised animal model of pulmonary fibrosis, there remains debate in the literature as to its usefulness as a model of IPF (Borzone *et al*., 2001; Chua *et al*., 2005). This debate has arisen as a result of a number of factors. Firstly, intra-tracheal administration of bleomycin produces a bronchiolocentric pattern of lung fibrosis. This is in contrast to IPF where a peripheral and sub-pleural pattern of fibrosis is seen (Scadding & Hinson 1967). However, intravenous or intra-peritoneal administration of bleomycin will induce pulmonary fibrosis in a peripheral and sub-pleural distribution (Adamson 1976; Baran *et al*., 2007), akin to IPF. The question therefore (to my mind) is not whether the pattern of fibrosis that bleomycin induces is absolutely comparable with IPF, but whether the fibrosis that bleomycin induces may be used as a tool to study the fibrosis of IPF. It is clear from over the years that the bleomycin model has informed considerable understanding of pulmonary fibrosis, of direct applicability to IPF, and it is for these reasons that bleomycin continues to be used.

A second aspect of the bleomycin model which often raises concern is that it induces significant inflammation that precedes fibrosis. This is in contrast to modern theories of IPF which propose that IPF develops in the absence of preceding inflammation (Selman *et al*., 2001; Gauldie 2002). However, a well recognised advantage of the bleomycin

model is its reproducibility (Chua *et al*., 2005). As such, once the model has been optimised in a research facility, the stage of progressive fibrosis can be defined and interrogated. This approach has become increasingly adopted, with investigators targeting this post-inflammatory fibrotic phase of the bleomycin model to gather information that has clear translational potential (Fichtner-Feigl *et al*., 2006; Chaudhary *et al*., 2007; Choi *et al*., 2009) with regard to fibrotic lung disease. It was using this premise that my depletional studies were designed. Whether inflammation precedes fibrosis in IPF is in itself a matter for much debate (Gauldie 2002; Strieter 2002; Stramer *et al*., 2007). The dominant theory in the lung community at present is that the fibrosis of IPF occurs in the absence of inflammation (or in the presence of minimal inflammation) (Selman *et al*., 2001), although some prominent respiratory researchers do still favour an inflammation-fibrosis link in IPF (Strieter 2002; Keane 2008).

A third aspect of the bleomycin model that raises concern is its durability (Borzone *et al*., 2001; Chua *et al*., 2005). Borzone and colleagues have questioned the usefulness of the bleomycin model as a model of lung fibrosis. In their paper they state that the fibrosis seen in the bleomycin model is bronchiolocentric rather than peripheral, as in IPF, a contention that has been discussed above. Furthermore, they state that the later stage of their bleomycin model (>30 days in rats) is characterised by emphysema. This is because the greatest difference in emphysema score between control mice and bleomycin-treated mice was present at 130 days. However, analysis of the data shows that in bleomycin-treated animals emphysema is present at 15 days and is similar to that seen at 130 days. The difference that they cite thus reflects a reduction in emphysema score in control animals rather than an increase in bleomycin-treated animals *per se*. Furthermore, they have used a parametric test to analyse data that should have been analysed by a non-parametric test, questioning the validity of the data. Furthermore, this researcher believes that any emphysema score has inherent problems; the mere act of fixing lungs can induce “emphysema” as a result of the inflation technique used. My own histology demonstrates that lungs can be over-inflated and thus induce artifactual emphysema-like changes. In addition, my own late stage bleomycin groups (day 136)

do not have gross emphysema-like changes compared to the earlier time points. As a result therefore, I have some concerns over how much genuine importance can be attributed to this much cited paper.

In summary therefore, I have established a model of pulmonary fibrosis that is reproducible, reversible, has clearly defined stages of pulmonary fibrosis, and which demonstrates the time-points at which the appearance of macrophages and myofibroblasts is most prominent.

Finally, I went on to use the AdTGFβ model of pulmonary fibrosis. I established that $1x10^8$ pfu of AdTGFβ was the optimal dose that would allow detection of AdTGFβ in BALF, induce significant fibrosis at 14 days, and not be harmful to mice. The AdTGFβ model has been used in several studies examining the pathogenesis of pulmonary fibrosis (Sime *et al*., 1997; Liu *et al*., 2001; Warshamana *et al*., 2002a; Washmen *et al*., 2002b; Ask *et al*., 2008). As mentioned previously, some researchers believe it is more resemblant of IPF as it induces lung fibrosis with minimal inflammation. However, as the virus has tropism for the bronchial and alveolar epithelium, then any pulmonary fibrosis that ensues is epithelial-dependent. This would favour the hypothesis that IPF is initiated through epithelial injury (Selman *et al*., 2001), but raises come concern over whether this model has general applicability to a more global lung injury, if that is what causes IPF. Regardless, using two well characterised models of pulmonary fibrosis should ensure that any findings are likely to reflect the true pathogenesis of pulmonary fibrosis, rather than being model-specific and it is for reason that I employed this strategy.

# CHAPTER 4

## LUNG MACROPHAGE REGULATION OF PULMONARY FIBROSIS

### *4.1 Abstract*

Although alternatively activated macrophages have substantive pro-fibrotic functions, the role of macrophages in pulmonary fibrosis remains undefined and controversial. Using *in vivo* depletional strategies I demonstrate that lung macrophages are key regulators of pulmonary fibrosis and exhibit differential effects during lung fibrogenesis as compared with lung fibrosis resolution. Specifically, depletion of lung macrophages during lung fibrogenesis leads to amelioration of lung fibrosis. In contrast, depletion of lung macrophages during fibrosis resolution leads to a persistence of lung fibrosis. Furthermore, I identify the alternatively activated macrophage phenotype as a key cellular regulator during lung fibrogenesis in a murine model of lung fibrosis, and show the presence of this macrophage phenotype in the archetypal human fibrotic lung disorder, idiopathic pulmonary fibrosis.

*4.2 Introduction*

Pulmonary fibrosis is the common end-point of many disorders of the lung such as the adult respiratory distress syndrome (ARDS), granulomatous disorders of the lung including sarcoidosis, occupational lung diseases such as asbestosis, and collagen vascular diseases with lung involvement such as rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosis (SLE) (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias). The archetypal fibrotic lung disease, idiopathic pulmonary fibrosis, is a devastating disorder with an unknown natural history (Gross *et al*., 2001); there are no effective therapies (Walter *et al*., 2006), its incidence is rising (Raghu *et al*., 2006) and it has a median survival of between 2.5-3.5 years (Nagai *et al*., 1999; King *et al*., 2000; Selman *et al*., 2001).

There has been much controversy regarding the role of macrophages in the pathogenesis of pulmonary fibrosis. Several papers have recently suggested their importance (Okuma *et al*., 2004; Mora *et al*., 2006; Prasse *et al*., 2006; Baran *et al*., 2007; Murray *et al*., 2010). Importantly, it has been demonstrated in rodents using the bleomycin model that lung macrophages are likely to be the predominant source of transforming growth factor-β (TGFβ) (Khalil *et al*., 1989), a most prominent fibrosis-inducing molecule. Furthermore it has been shown in a model of lung fibrosis that corticosteroid treatment has no effect on alveolar macrophage secretion of TGFβ, and that when alveolar macrophages from bleomycin treated animals are cultured in the presence of corticosteroids there is in fact increased secretion of TGFβ (Khalil *et al*., 1993). Moreover, a recent study in patients who develop an accelerated form of fibrotic lung disease demonstrated that they have dysregulated alveolar macrophages (Rouhani *et al*., 2009).

Functional activation states of macrophages are associated with fibrogenic phenotypes in mice and humans (Mantovani *et al*., 2002; Gordon 2003; Varin & Gordon 2009). In

solid organs such as the liver and kidney, the critical role of the macrophage in regulating fibrosis is well established (Duffield *et al*., 2005; Henderson *et al*., 2008).

I sought to determine whether lung macrophages are involved in the pathogenesis of lung fibrosis. Using the bleomycin and adenoviral-TGFβ models of lung injury and fibrosis in mice, I assessed the effects of depletion of lung macrophages during both the early and progressive phases of lung fibrogenesis and during the resolution phase of lung fibrosis. My work demonstrates that lung macrophages are required for both lung fibrogenesis and fibrosis resolution. Furthermore, I demonstrate the importance of the alternatively activated lung macrophage phenotype during lung fibrogenesis in mice, and show that they are present in patients with IPF.

*4.3 Results*

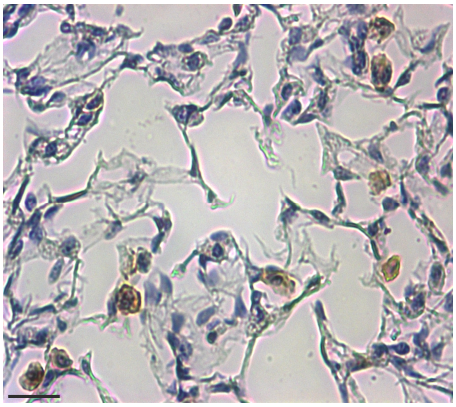
**Liposomal clodronate depletes lung macrophages**

Administration of liposomal clodronate (dichloromethylene bisphosphonate or $Cl_2MBP$) is a well established method of selectively and effectively depleting macrophages (Getts *et al*., 2008; Jaiswal *et al*., 2009; Zhang *et al*., 2009). It is selectively taken up by phagocytes which subsequently die of apoptosis, the so called liposome-mediated macrophage 'suicide' approach (van Rooijen *et al*., 1994; van Rooijen *et al*., 1996). Liposomal clodronate has been shown to effectively deplete lung macrophages (Thepen *et al*., 1989; Holt *et al*., 1993; Manicone *et al*., 2009). Peak depletion occurs after 24 hours, but persists for up to 5 days (Thepen *et al*., 1989).

I administered liposomal clodronate (100µl) by i.t. route to 0.9% saline-treated female C57Bl/6 mice and show by immunohistochemistry that lung macrophages are effectively depleted (Figure 4.1, A and B). Lung macrophages were stained using the macrophage-restricted marker, F4/80. To establish whether administration of liposomal clodronate i.t. depletes lung macrophages in the context of bleomycin injury, female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0, liposomal clodronate (100µl) i.t. on day 21 and culled on day 22. I show that lung macrophages are effectively depleted (Figure 4.1, C, D and E) and using a semi-quantitative score I established that the efficiency of depletion was approximately 70% (Figure 4.1E).

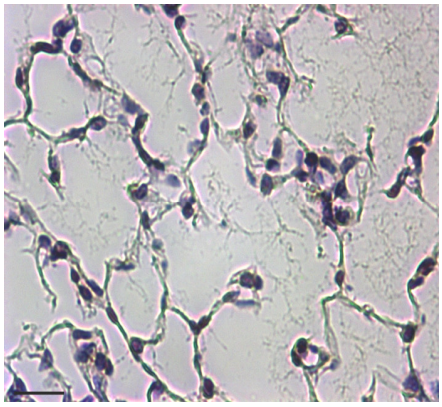
# Figure 4.1

A



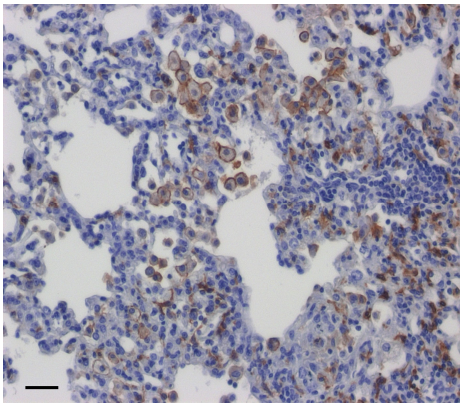
0.9% saline

B



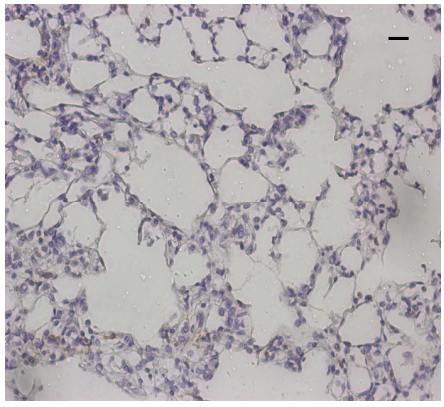
0.9% saline + LC

C



Bleomycin

D



Bleomycin + LC

E

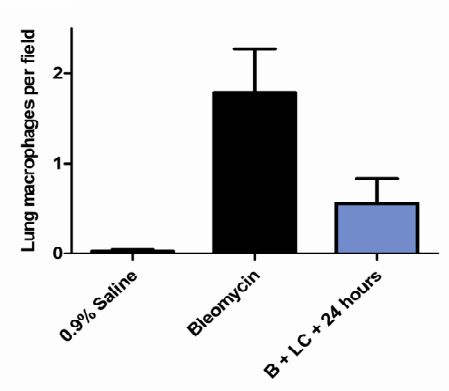


Figure 4.1

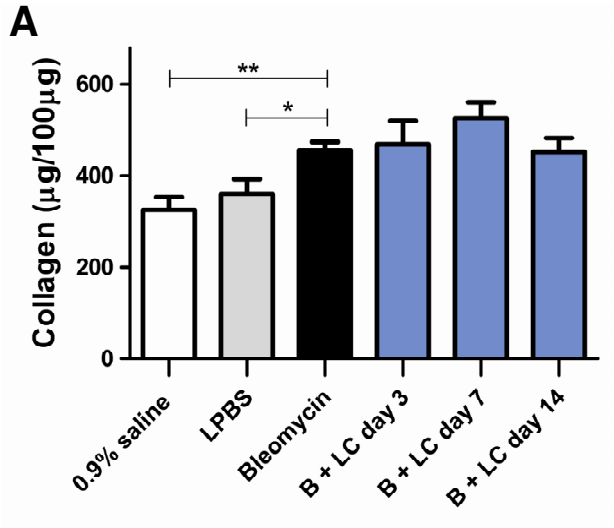
**Liposomal clodronate depletes lung macrophages**

Female C57Bl/6 mice were given i.) 0.9% saline i.t. (50µl) +/- liposomal clodronate (LC) i.t. (100µl) and culled 24 hours later, or, ii) bleomycin i.t. (50µl) +/- liposomal clodronate i.t. (100µl) on day 21 and culled 24 hours later. Immunohistochemistry (IHC) with F4/80 antibody staining was performed on lung sections (A)-(D), and quantified (E). (A) 0.9% saline i.t. alone showing lung macrophages stain with F4/80 antibody. (B) 0.9% saline *plus* LC i.t. demonstrates that lung macrophages are effectively depleted. (C) Bleomycin i.t. alone showing lung macrophages stain with F4/80 antibody. (D) Bleomycin *plus* LC i.t. demonstrates that lung macrophages are effectively depleted. (E) Mice were given 0.9% saline or bleomycin (B) alone and culled on day 22, or B *plus* LC (day 21) and culled on day 22. Quantification of F4/80 positive cells per field was performed. There was a 70% reduction in the number of lung macrophages following LC administration. n=3-4. Bar 20µm.

**Lung macrophages are not necessary for the inflammation initiation of fibrosis**

To assess the role of lung macrophages during the inflammatory phase of bleomycin-induced lung fibrosis, I administered a single i.t. instillation of 0.033mg bleomycin and culled mice after 18 days. Liposomal clodronate (100µl) was given i.t. on either day 3, 7 or 14. Additional controls of 0.9% saline (50µl) and liposomal PBS (100µl) were given i.t. on day 0 and mice culled on day 18. As shown in Figure 4.2A, depletion of lung macrophages at any of the time-points has no effect on the inflammatory phase and early induction of lung fibrosis as measured by Sircol assay. This lack of effect was confirmed by Masson's trichrome staining on lung sections as can be seen in representative images shown in Figure 4.2, B-G.

# Figure 4.2


A
Collagen (µg/100µg)
600
400
200
0
**
*
0.9% saline
LPBS
Bleomycin
B + LC day 3
B + LC day 7
B + LC day 14

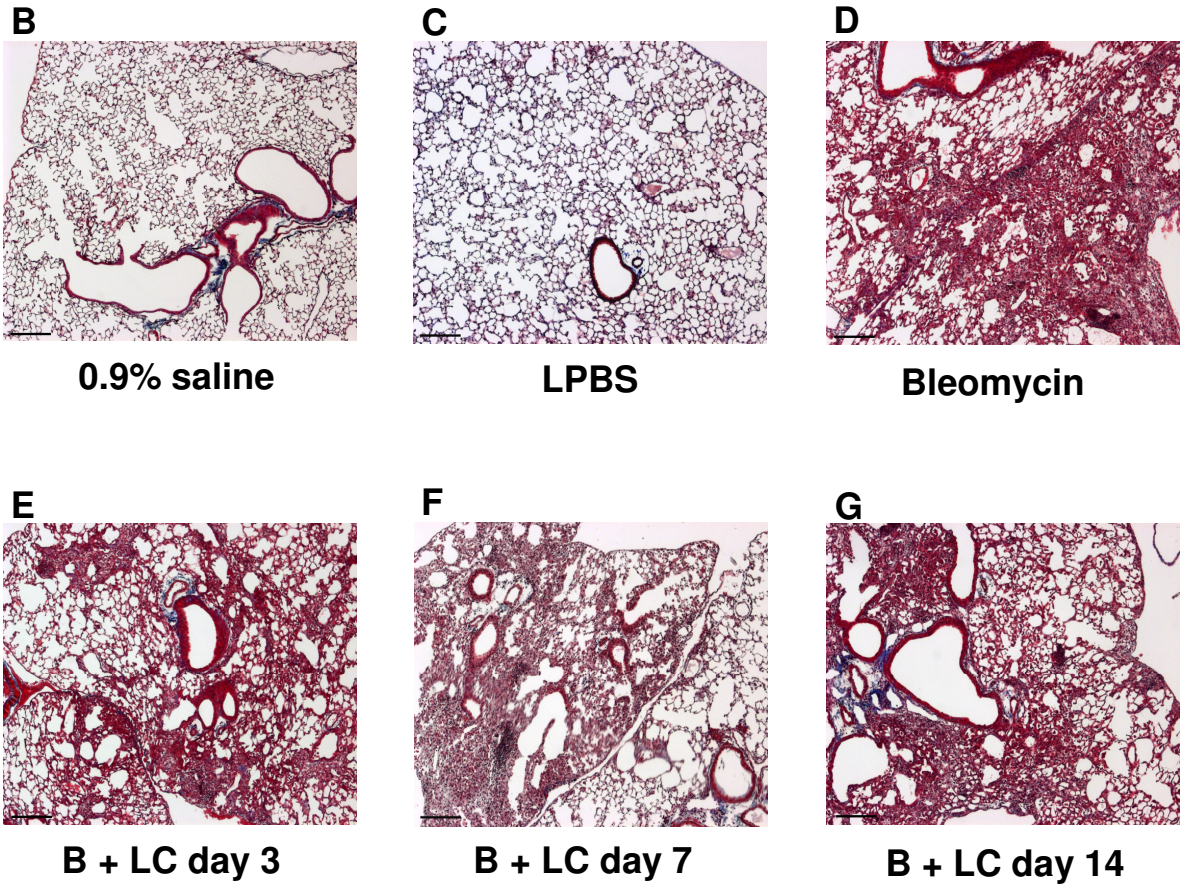

B
0.9% saline
C
LPBS
D
Bleomycin
E
B + LC day 3
F
B + LC day 7
G
B + LC day 14

Figure 4.2

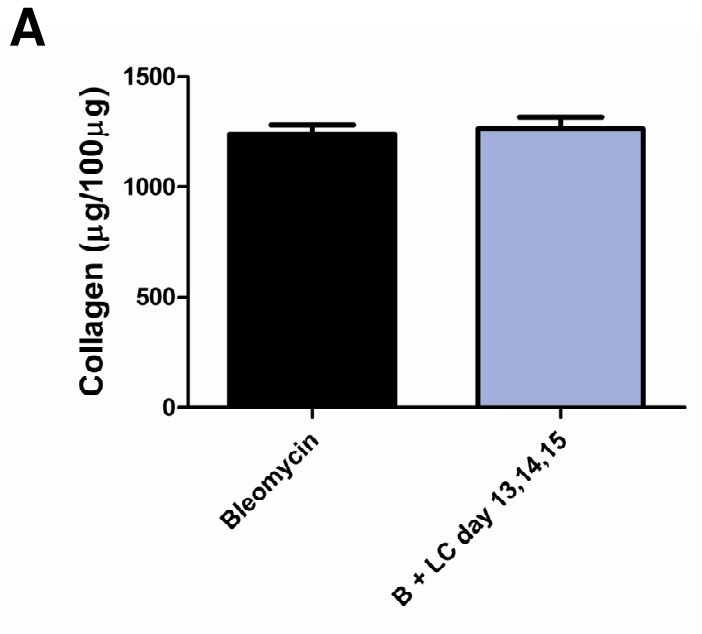
**Lung macrophages are not necessary for the inflammation initiation of fibrosis**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 18. In addition to bleomycin, groups of mice were also given liposomal clodronate (LC) i.t. (100µl) at various time-points. All mice were culled on day 18. Fibrosis was measured by Sircol assay. 0.9% saline (50µl) and liposomal control (liposomal phosphate buffered saline, LPBS (100µl)) were given i.t. as controls and mice culled on day 18. Significant differences in fibrosis were found between bleomycin and controls (vs. saline, **p=0.0012; vs. LPBS *p=0.0160), but there were no differences when LC was given in combination with bleomycin. (B)-(G) Masson's trichrome staining on representative lung sections of mice treated with: (B) 0.9% saline alone, (C) LPBS alone, (D) Bleomycin alone, (E) Bleomycin *plus* LC i.t. on day 3, (F) Bleomycin *plus* LC i.t. on day 7, (G) Bleomycin *plus* LC i.t. on day 14. n=6-8 per group. Data are presented as mean +/- SEM. Bar 200µm.

**Depletion of lung macrophages during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on subsequent pulmonary fibrosis**

To assess whether macrophages present during the inflammatory phase of bleomycin-induced lung fibrosis play a role in the subsequent development of pulmonary fibrosis, I administered a single i.t. instillation of 0.033mg bleomycin and culled mice after 32 days. Liposomal clodronate (100µl) was given i.t. on three consecutive days: 13, 14 and 15. As shown in Figure 4.3A, depletion of lung macrophages during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on the subsequent development of pulmonary fibrosis, as measured by Sircol assay. This lack of effect was confirmed by Masson's trichrome staining on lung sections as can be seen in representative images shown in Figure 4.3, B and C.

# Figure 4.3


A
1500
1000
500
0
Collagen (µg/100µg)
Bleomycin
B + LC day 13,14,15

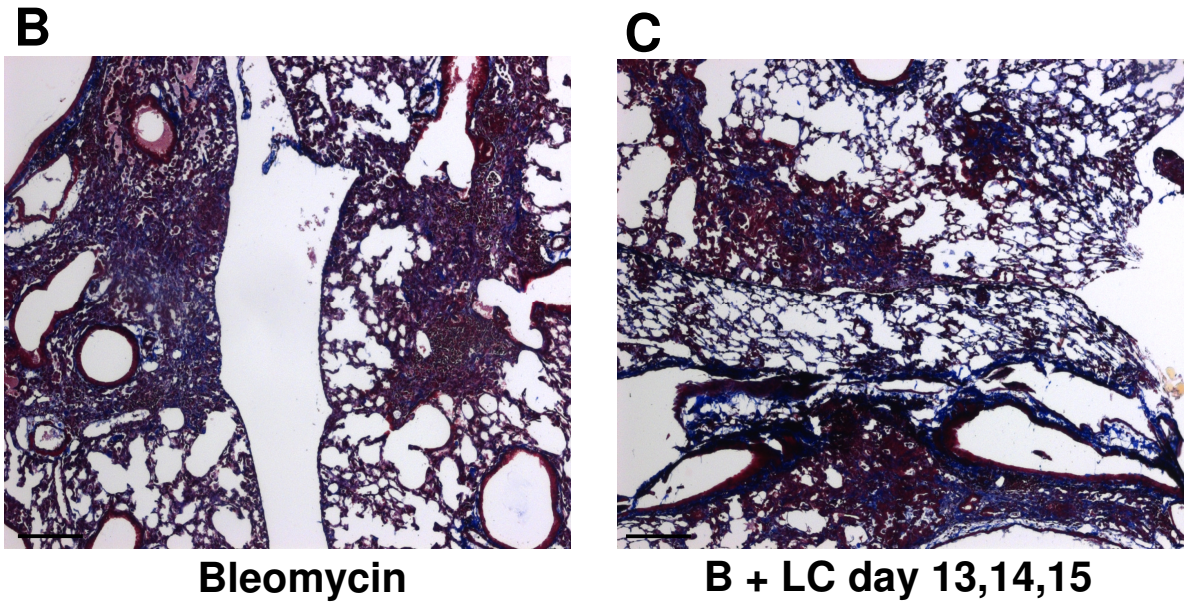

B
C
Bleomycin
B + LC day 13,14,15

Figure 4.3

**Depletion of lung macrophages during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on subsequent pulmonary fibrosis**

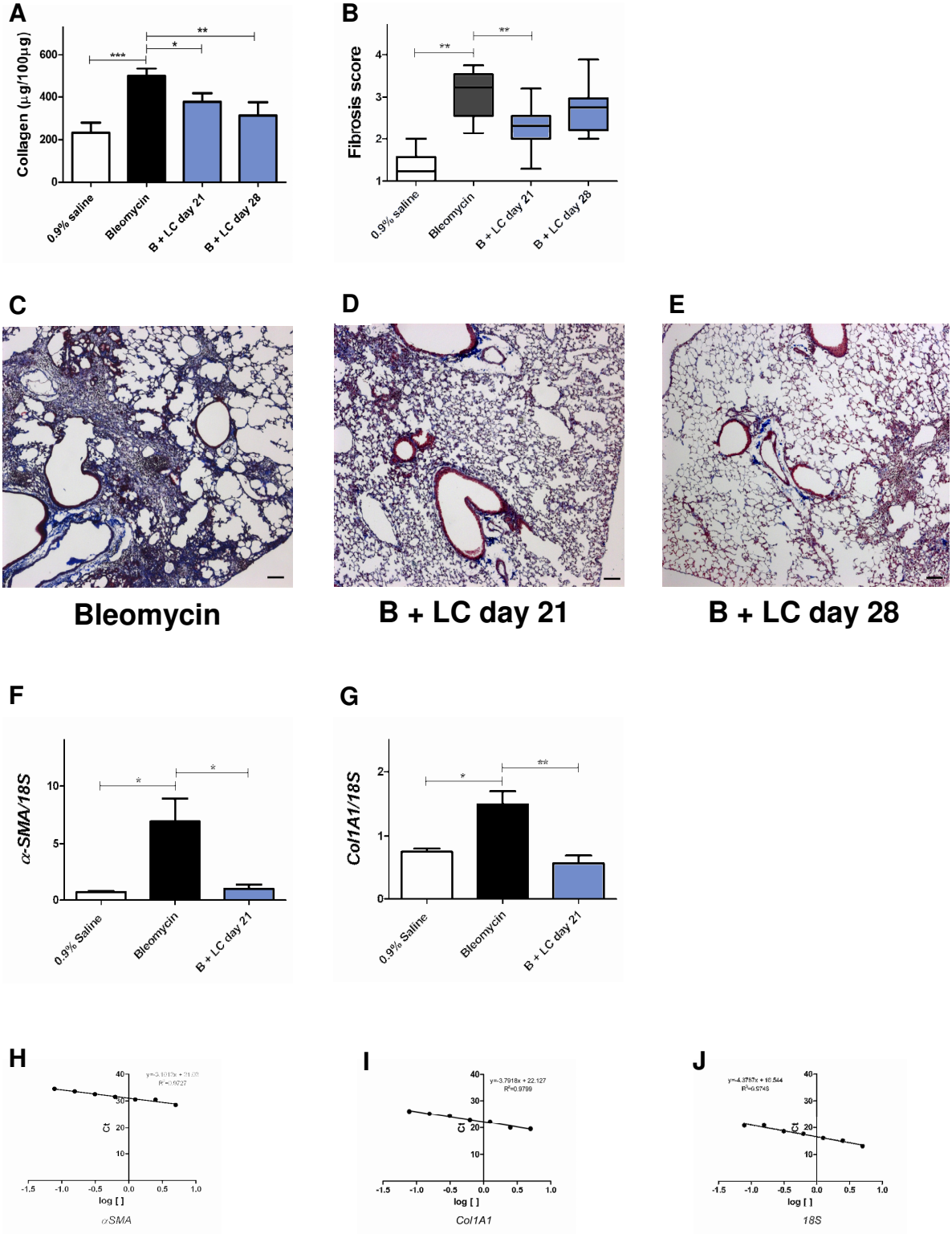
Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl). In addition to bleomycin, a group of mice were also given liposomal clodronate (LC) i.t. (100µl) on three consecutive days: 13, 14 and 15. All mice were culled on day 32. Fibrosis was measured by Sircol assay. (A) No difference in fibrosis was found following lung macrophage depletion (bleomycin vs. bleomycin, B, *plus* LC on days 13, 14, 15, p=ns). (B) and (C) Masson's trichrome staining on representative lung sections of mice treated with: (B) bleomycin alone or (C) bleomycin *plus* LC i.t. on days 13, 14, 15. n=6 per group. Data are presented as mean +/- SEM. Bar 200µm.

**Lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model**

Having established that macrophages appear to have no role in the inflammation initiation of fibrosis, and that depletion of macrophages during the inflammatory phase has no effect on subsequent on pulmonary fibrosis in the bleomycin model, I next wished to evaluate the role of lung macrophages during the progressive fibrotic phase of pulmonary fibrosis (day 18 onwards). In my bleomycin model, fibrosis peaks at 32 days (Chapter 3, Figure 3.5, A, B and G). Consequently, female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0 and culled on day 32. Lung macrophages were depleted by giving liposomal clodronate (100μl) as a single instillation i.t. on either day 21 or day 28. As shown in Figure 4.4A, depletion of lung macrophages at both time points significantly reduces the degree of pulmonary fibrosis that develops as measured by Sircol assay. It has previously been shown that this effect is not due to apoptosis per se because rats given apoptotic macrophages (Wang *et al*., 2003) or apoptotic cells (Wang *et al*., 2006) actually develop lung fibrosis. However, to ensure that this result was mediated by lung macrophage depletion *per se* rather than being a liposomal effect, I set up an independent experiment where female C57Bl/6 mice were given 0.033mg bleomycin i.t. alone or 0.033mg bleomycin i.t. on day 0 plus liposomal phosphate-buffered saline (LPBS) i.t. (100μl) on day 21, and culled on day 32. As shown in Figure 4.4, K, L, and M there was no reduction in fibrosis after administration of LPBS.

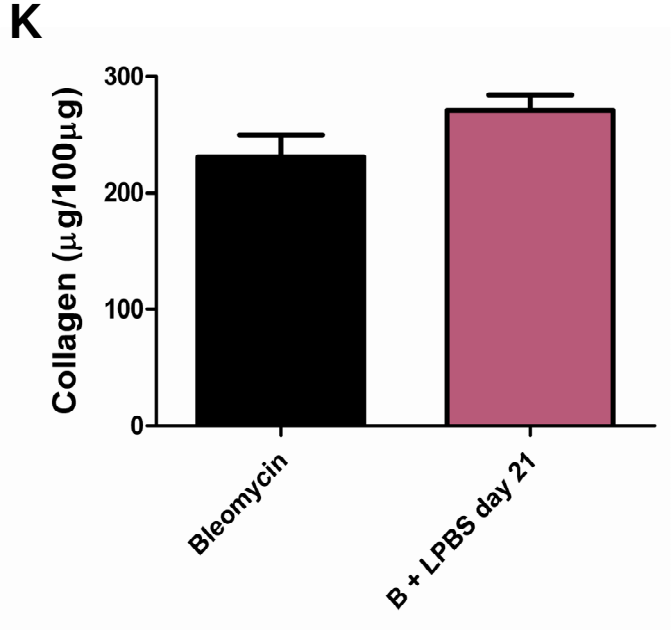
Reduction in pulmonary fibrosis following lung macrophage depletion was confirmed by semi-quantitative fibrosis scoring of lung sections stained by Masson's trichrome (Figure 4.4, B, C, D and E). Furthermore, quantitative PCR (qPCR) performed on lung homogenates 25 days after bleomycin instillation demonstrated a reduction in expression of the surrogate markers of fibrosis *α–smooth muscle actin* (*α-SMA*) and *Col1A1* after depletion of lung macrophages (on day 21) (Figure 4.4, F and G, respectively). Standard curves for the qPCR are shown in Figure 4.4, H-J.

# Figure 4.4.1

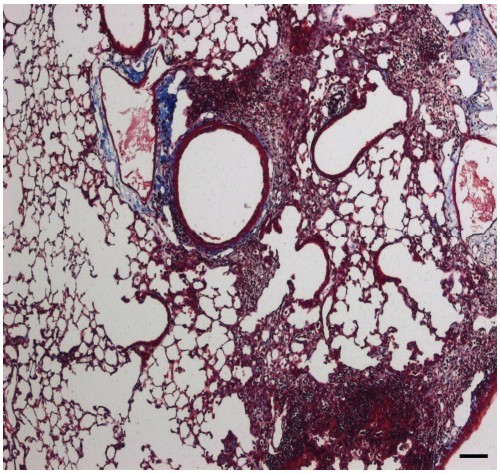

A
Collagen (µg/100µg)
0
200
400
600
***
*
**
0.9% saline
Bleomycin
B + LC day 21
B + LC day 28
B
Fibrosis score
1
2
3
4
**
**
0.9% saline
Bleomycin
B + LC day 21
B + LC day 28
C
D
E
Bleomycin
B + LC day 21
B + LC day 28
F
α-SMA/18S
0
5
10
*
*
0.9% Saline
Bleomycin
B + LC day 21
G
Col1A1/18S
0
1
2
*
**
0.9% Saline
Bleomycin
B + LC day 21
H
Ct
log [ ]
αSMA
I
Ct
log [ ]
Col1A1
J
Ct
log [ ]
18S

# Figure 4.4.2

K

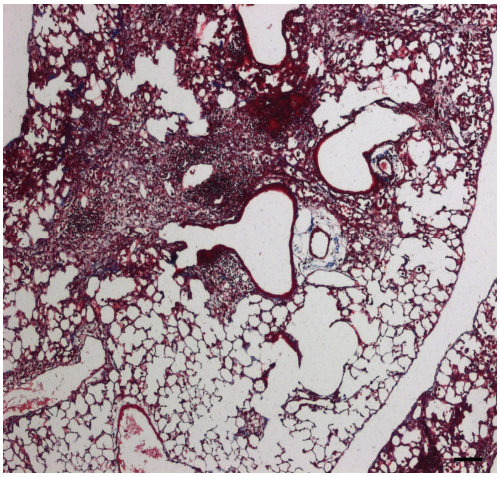



L



Bleomycin

M



B + LPBS day 21

Figure 4.4

**Lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) and fibrosis quantified (A), (B). 0.9% saline was given i.t. (50µl) as a control. Bleomycin 0.033mg (50µl) +/- liposomal control (LPBS) i.t. (100µl) were administered to female C57Bl/6 mice in an independent experiment (H)-(J). (A), (B) Mice were given 0.9% saline, bleomycin alone, or bleomycin, B, *plus* liposomal clodronate (LC) i.t. (100µl) during progressive fibrosis and culled at peak lung fibrosis (day 32). Depletion of lung macrophages reduces lung fibrosis as measured by Sircol assay (B alone vs. B *plus* LC (day 21), *p=0.0247; B vs. B plus LC (day 28), **p=0.0087). (B) Depletion of lung macrophages reduces lung fibrosis as quantified by fibrosis score (B vs. B *plus* LC (day 21), **p=0.0051; B vs. B *plus* LC (day 28), trend to reduction). (C)-(E) Masson's trichrome staining on representative lung sections of mice treated with bleomycin (C), B *plus* LC (day 21) (D) or B *plus* LC (day 28) (E). (F) and (G) Female C57Bl/6 mice were given bleomycin 0.033mg (50µl) +/- LC i.t. (100µl) on day 21 and culled on day 25. qPCR was performed on lung homogenates. (F) Depletion of lung macrophages leads to a reduction in *α-SMA* gene expression, *p=0.0349. (G) Depletion of lung macrophages leads to a reduction in *Col1A1* gene expression, **p=0.0083. (H)-(J) Representative standard curves used in qPCR analysis are shown for (H) *αSMA*, bleomycin treated lung, (I) *Col1A1*, bleomycin treated lung, (J) *18S*, bleomycin treated lung. (K)-(M) Female C57Bl/6 mice were given bleomycin alone, or bleomycin, B, *plus* LPBS i.t. (100µl) on day 21 and culled on day 32. (K) Administration of bleomycin *plus* liposomal control does not reduce fibrosis as measured by Sircol assay. (L)-(M) Masson's trichrome staining on representative lung sections of mice treated with bleomycin (L) or bleomycin, B, *plus* LPBS (day 21) (M). (A)-(E) n=6-15. (F)-(G) n=3-5. (K)-(M) n=5 Data are presented as (A), (F), (G), (K) mean +/- SEM, and, (B) Box and whiskers (Median, Inter-quartile range, minimum to maximum). Bar 100µm.

**Lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis in the AdTGFβ model**

Having established that lung macrophages regulate the progressive fibrotic phase of bleomycin-induced pulmonary fibrosis, I wished to back up my findings using a second model. As a result I next sought to determine the effect of depletion of lung macrophages during the progressive fibrotic phase of fibrosis in the AdTGFβ model. Previous work using this model in mice has established that peak fibrosis occurs at around 14 days (Warshamana et al., 2002). My dose finding study (Chapter 3, Figure 3.6B) identified $1x10^8$ pfu/50μl as the optimal dose of AdTGFβ that could be given to induce significant lung fibrosis without causing harm. As a result female C57Bl/6 mice were given $1x10^8$ pfu (50μl) of AdTGFβ i.t. on day 0 and culled on day 14. In addition mice were given liposomal clodronate (100μl) i.t. on day 10. Empty adenoviral vector (AdDL70-3, $1x10^8$ pfu (50μl)) was given i.t. as a control and did not induce fibrosis (Figure 4.5, A and B). Depletion of lung macrophages leads to a reduction in pulmonary fibrosis as measured by Sircol assay (Figure 4.5A). This was confirmed by Masson's trichrome staining of lung sections as can be seen in representative images shown in Figure 4.5, C and D. These results back up my findings in the bleomycin model and further suggest that lung macrophages are important in promoting the progressive phase of pulmonary fibrosis.

# Figure 4.5

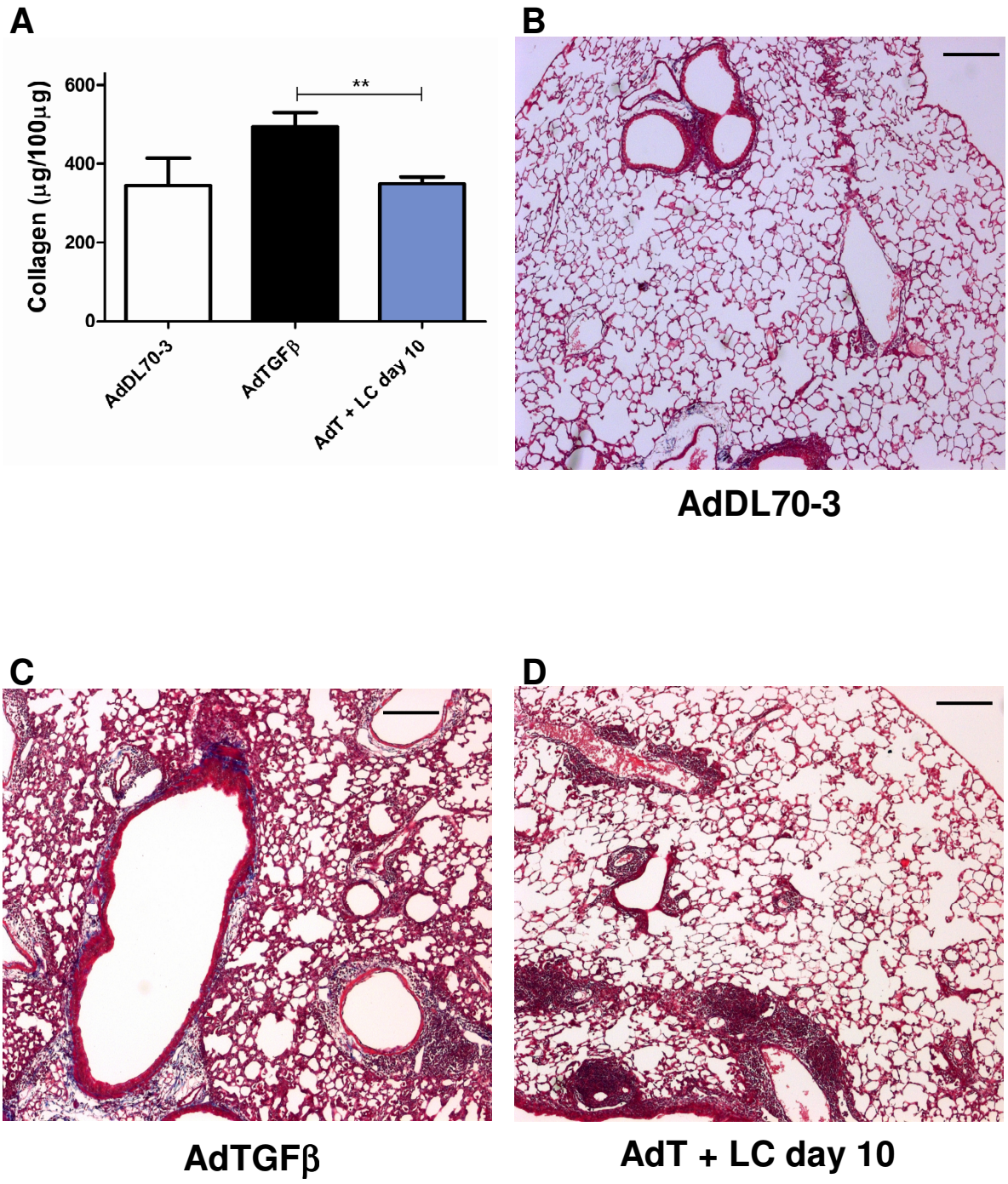

A
B
C
D
Collagen (µg/100µg)
600
400
200
0
**
AdDL70-3
AdTGFβ
AdT + LC day 10
AdDL70-3
AdTGFβ
AdT + LC day 10

Figure 4.5

**Lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis in the AdTGFβ model**

Female C57Bl/6 mice were given $1x10^8$ pfu of AdTGFβ i.t. (50μl) and fibrosis quantified by Sircol assay (A). AdDL70-3 ($1x10^8$ pfu/50μl) was given as a control. Masson's trichrome staining was performed on lung sections (B)-(D). (A) Mice were given AdDL70-3, AdTGFβ alone or AdTGFβ (AdT) *plus* liposomal clodronate (LC) i.t. (100μl) on day 10 and culled at peak lung fibrosis (day 14). Depletion of lung macrophages reduces lung fibrosis as measured by Sircol assay (AdTGFβ alone vs. AdT *plus* LC (day 10), **p=0.0079). (B) AdDL70-3 does not induce significant fibrosis at 14 days. (C) AdTGFβ induces significant fibrosis by 14 days. (D) Depletion of lung macrophages ameliorates lung fibrosis. (A)-(D) n=4-5. Data are presented as mean +/- SEM. Bar 200μm.

**CD11c$^+$ cells regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model**

As the role of macrophages in pulmonary fibrosis has been controversial for such a long time, I wanted to be absolutely sure that my previous findings truly indicated the importance of macrophages in the pathogenesis of pulmonary fibrosis. Consequently, I sought to establish the role of macrophages in pulmonary fibrosis using a different and independent lung macrophage depletional strategy. As a result I used CD11c-DTR transgenic mice. Their generation has previously been described (Hochweller *et al*., 2008). They were kindly supplied by Dr Andrew MacDonald (Institute of Immunology and Infection Research, University of Edinburgh) and Professor Gunter Hammerling (Deutsches Krebsforschungszentrum (DKFZ, German Cancer Research Centre), Heidelberg). Administration of diphtheria toxin (DT) i.t. to this transgenic mouse is known to deplete CD11c$^+$ cells (Landsman *et al*., 2007; van Rijt et al., 2005). In the lung interstitium, the major CD11c$^+$ cell type is the interstitial macrophage, dendritic cells being the minor component (Landsman *et al*., 2007). Within the alveolar space alveolar macrophages represent 80-90% of the CD11c$^+$ cell population (Landsman *et al*., 2007), the remainder being dendritic cells (Landsman *et al*., 2007; van Rijt *et al*., 2005). The major effect of the CD11c-DTR transgenic system therefore is the depletion of lung macrophages, both interstitial and alveolar. It also depletes dendritic cells, however they represent in absolute numbers the minor component of the lung CD11c$^+$ cell population. Furthermore, it has been argued that dendritic cells are "immature macrophages" and simply represent "a heterogeneous subset of mononuclear phagocytes [macrophages]" (Hume 2008), and as such it could be argued (especially in the lung) that all CD11c$^+$ cells are a subset of macrophages. Administration of DT depletes interstitial macrophages for up to 9 days and alveolar macrophages for 2-4 days (Landsman *et al*., 2007). For this reason I used the CD11c-DTR transgenic system as another means of evaluating the effect of depletion of lung macrophages on lung fibrogenesis.

I assessed the effect of depletion of lung macrophages on the degree of lung fibrosis using the bleomycin model. Initially I assessed the efficiency of CD11c$^+$ cell depletion,

using DT, by performing flow cytometric analysis on cells from bronchoalveolar lavage (BAL) fluid: female CD11c-DTR mice were given 0.033mg bleomycin i.t. followed by DT i.t. (8ng/g, 50µl) on day 21. Mice were culled on day 22. As shown in Figure 4.6, D-H administration of DT results in a greater than 90% reduction in the number of $CD11c^+$ cells in BALF. Next female CD11c-DTR mice were given 0.033mg bleomycin i.t. on day 0 +/- DT (8ng/g, 50µl) i.t. on day 21, and culled on day 32 to assess the effect on lung fibrosis. As demonstrated in Figure 4.6A depletion of lung macrophages results in a significant reduction in lung fibrosis as measured by Sircol assay. Masson's trichrome staining on lung sections confirmed this finding as can be seen in representative images shown in Figure 4.6, B and C.

.

# Figure 4.6

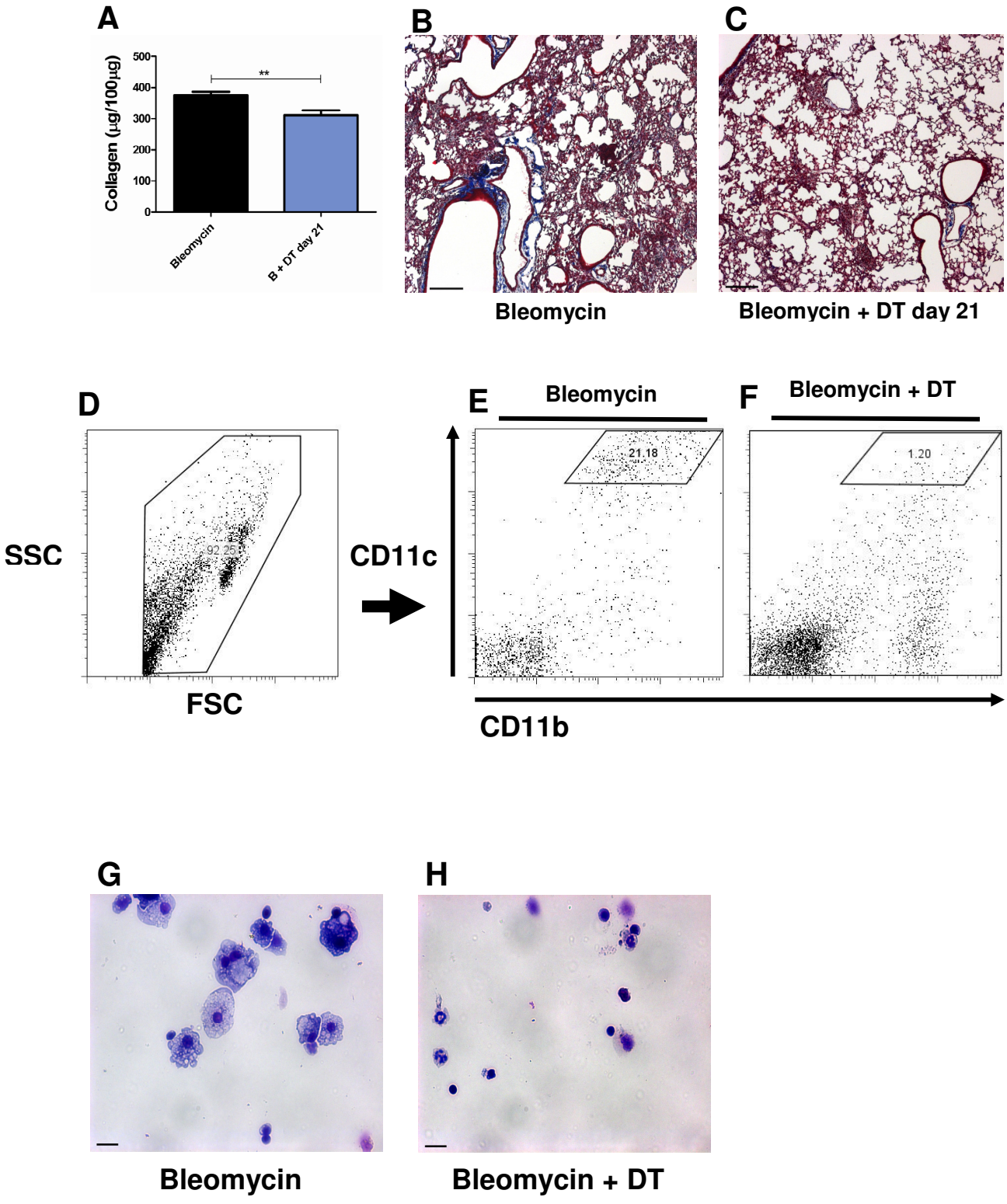

A
B
C
Collagen (µg/100µg)
500
400
300
200
100
0
**
Bleomycin
B + DT day 21
Bleomycin
Bleomycin + DT day 21
D
E
F
Bleomycin
Bleomycin + DT
SSC
FSC
92.25
CD11c
CD11b
21.18
1.20
G
H
Bleomycin
Bleomycin + DT

Figure 4.6

**CD11c$^+$ cells regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model**

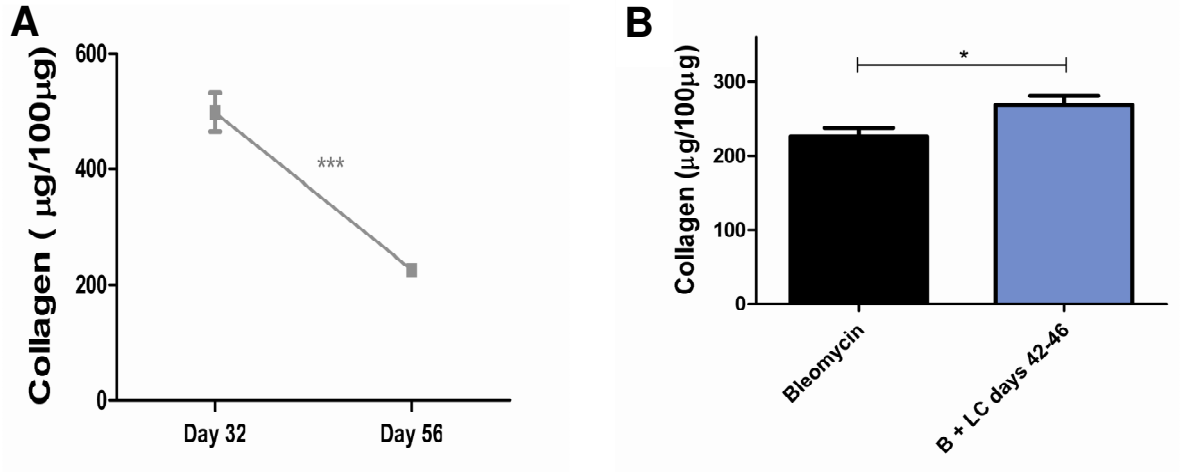
Female CD11c-DTR mice were given 0.033mg bleomycin i.t. (50µl) +/- diphtheria toxin (DT) 8ng/g i.t. (50µl) on day 21 and culled on day 32 (A)-(C) or day 22 (D)-(H). (A) Depletion of lung macrophages ameliorates lung fibrosis as measured by Sircol assay (Bleomycin (B) alone vs. B *plus* DT, **p=0.0044). (B) and (C) Masson's trichrome staining on representative lung sections of mice treated with B alone (B), or B *plus* DT (C). Bronchoalveolar lavage (BAL) cells were taken and analysed by flow cytometry (D)-(F) or cytospins created (G)-(H). BAL cells were stained with anti-CD11b:FITC, IAb:PE and CD11c:APC. DT administration depletes BAL macrophages by >90% (E)-(F). BAL macrophages (G) are replaced by mononuclear cells and granulocytes following DT administration (H). (A)-(C) n=6-7, (D)-(H) n=2 per group. Data are presented as mean +/- SEM. Bar 200µm (B)-(C), 20µm (G)-(H).

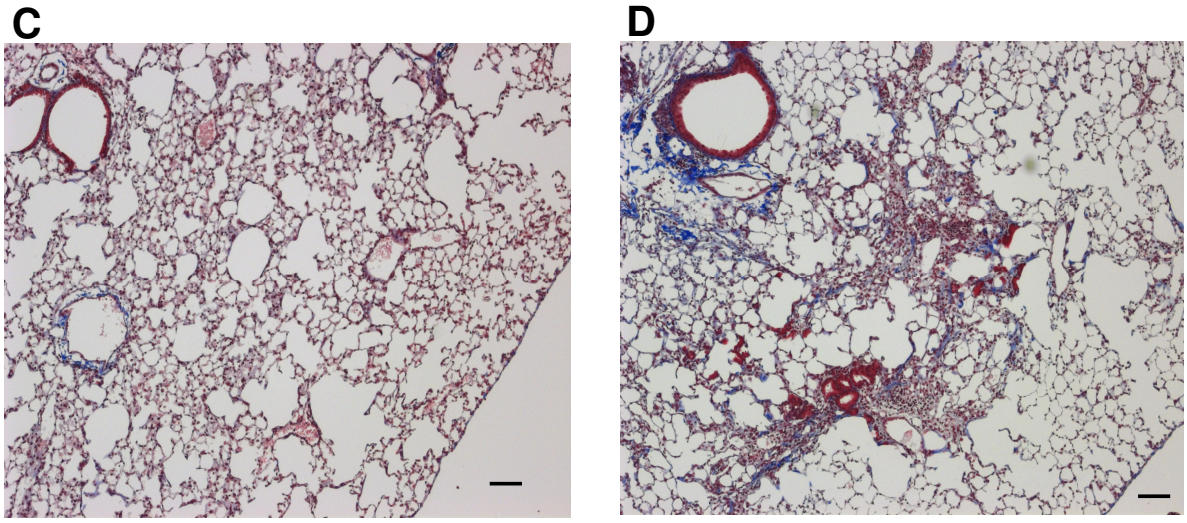
**Lung macrophages regulate the resolution phase of pulmonary fibrosis**

Previous work from our own group in an experimental model of liver fibrosis has demonstrated the distinct functions that macrophages exhibit during liver fibrosis progression and resolution (Duffield *et al*., 2005). It has been known for some time that the bleomycin model exhibits reversibility in its later stages (Izbicki *et al*., 2002; Moore *et al*., 2008) but this has not been intensely studied. I have shown already in my model that pulmonary fibrosis peaks around 32 days (Chapter 3, Figure 3.5, A, B and G). When I extended the time course I established that fibrosis had significantly resolved by 56 days (Chapter 3, Figure 3.2, A and H; Chapter 3, Figure 3.5, A and H). I therefore aimed to elucidate the role of lung macrophages during the resolution phase of bleomycin-induced pulmonary fibrosis.

Female C57Bl/6 mice were given bleomycin 0.033mg i.t. on day 0, with or without liposomal clodronate (100µl) i.t. daily on days 42-46. Mice were culled on day 56. As shown in Figure 4.7B, depletion of lung macrophages leads to a slowing of the resolution of fibrosis. Masson's trichrome staining of lung sections confirms this effect as can be seen in representative images shown in Figure 4.7, C and D.

# Figure 4.7




Bleomycin

B + LC days 42-46

Figure 4.7

**Lung macrophages regulate the resolution phase of pulmonary fibrosis**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 32 or 56 (A). In addition a group of mice were given bleomycin *plus* liposomal clodronate (LC) i.t. (100µl) daily on days 42-46 and culled on day 56 (B). Fibrosis was measured by Sircol assay. Masson's trichrome staining was performed on lung sections (C)-(D). (A) There is significant reversibility of lung fibrosis between day 32 and day 56 (bleomycin day 32 vs. bleomycin day 56, ***$p<0.001$). (B) Depletion of lung macrophages during the recovery phase slows the resolution of lung fibrosis (bleomycin alone vs. bleomycin, B, *plus* LC on days 42-46, *$p<0.05$). (C) & (D) Masson's trichrome staining of representative lung sections demonstrates resolution of lung fibrosis (C), which is attenuated following lung macrophage depletion (D). (A) n=7-15, (B)-(D) n = 7. Data are presented as mean +/- SEM. Bar 100µm.

**Alternatively activated macrophages characterise the progressive fibrotic phase of pulmonary fibrosis in mice**

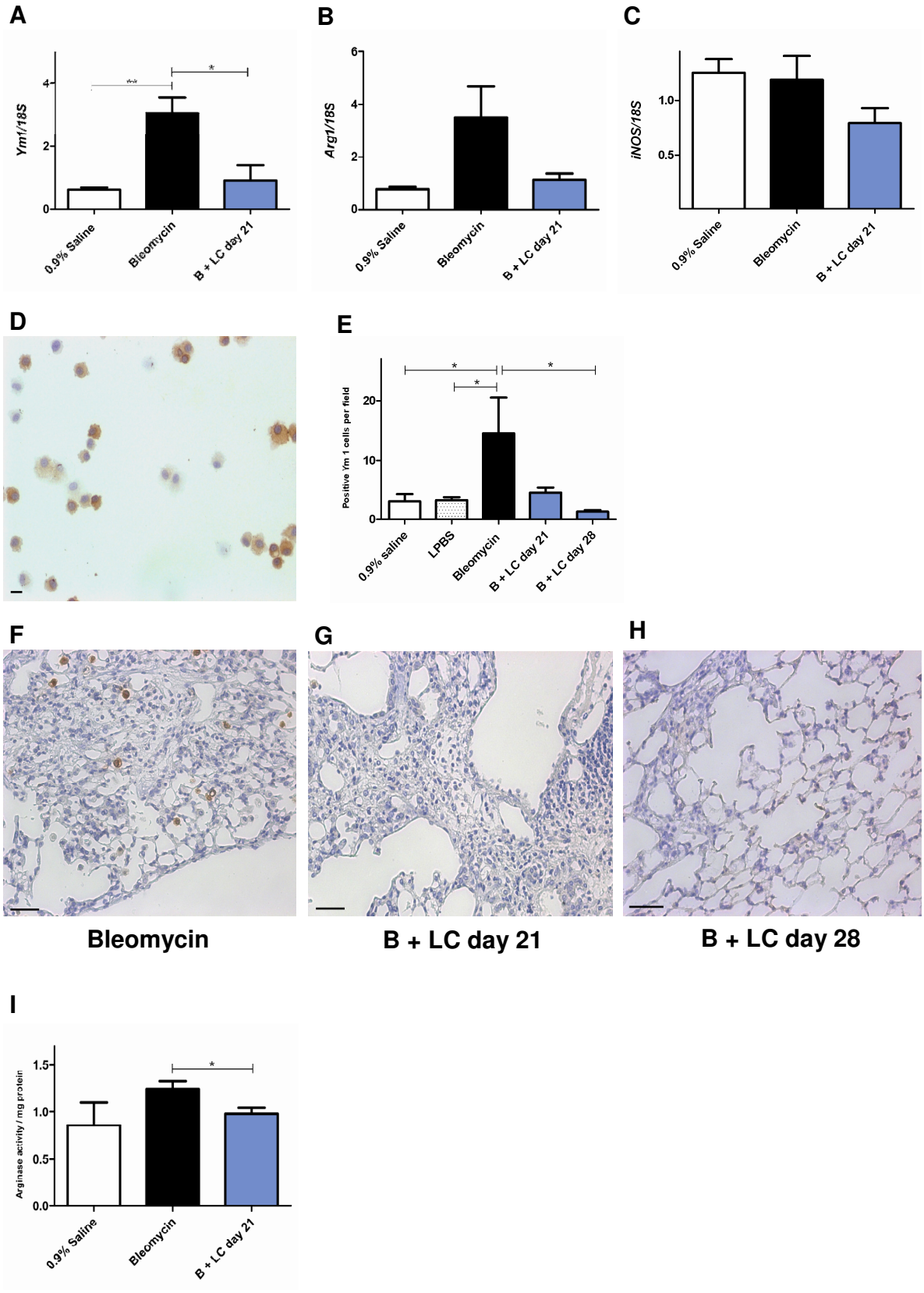
Several macrophage phenotypes have now been broadly described (Gordon & Taylor 2005). I have demonstrated that lung macrophages regulate the progressive phase of pulmonary fibrosis. As alternatively activated macrophages are believed to have pro-fibrotic potential I wished to establish whether it is the alternatively activated macrophage phenotype that characterises this lung macrophage fibrosis-promoting effect.

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0 with or without liposomal clodronate (100µl) i.t. on day 21, and culled on day 25. qPCR was performed on lung homogenates for markers of alternative macrophage activation. As shown in Figure 4.8, A and B, depletion of lung macrophages leads to a reduction in gene expression of characteristic markers of alternative macrophage activation, *Ym1* and *Arginase1*, respectively. Interestingly, the gene expression of *iNOS*, the prototypical marker of classical macrophage activation, is not affected by bleomycin administration and is not significantly reduced by lung macrophage depletion (Figure 4.8C). This data suggests that it may be the alternatively activated lung macrophage that regulates the progressive phase of lung fibrosis. However it is clear that more work is required to provide more concrete evidence of this.

Having established a possible effect at the RNA level, I wished to establish whether this effect was also seen at the protein level. Female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0, with or without liposomal clodronate (100µl) i.t. on either day 21 or day 28, and culled on day 32. Immunocytochemistry on BAL fluid cells demonstrates that following bleomycin administration, lung macrophages exhibit variable levels of protein expression of Ym1 (Figure 4.8D). Quantitative immunohistochemical analysis on lung sections demonstrated a reduction in Ym1 protein expression following lung macrophage depletion (Figure 4.8, E, F, G and H). Moreover, in an independent experiment female C57Bl/6 mice were given 0.033mg

bleomycin i.t. on day 0, with or without liposomal clodronate (100µl) i.t. on day 21 and culled on day 22. An arginase activity assay was performed on lung homogenates. Following lung macrophage depletion there was a reduction in arginase activity as shown in Figure 4.8I in keeping with the qPCR results. Standard curves for the qPCR analyses are shown in Figure 4.8, J-L.

# Figure 4.8.1


A
Ym1/18S
0.9% Saline
Bleomycin
B + LC day 21
B
Arg1/18S
0.9% Saline
Bleomycin
B + LC day 21
C
iNOS/18S
0.9% Saline
Bleomycin
B + LC day 21
D
E
Positive Ym 1 cells per field
0.9% saline
LPBS
Bleomycin
B + LC day 21
B + LC day 28
F
G
H
Bleomycin
B + LC day 21
B + LC day 28
I
Arginase activity / mg protein
0.9% Saline
Bleomycin
B + LC day 21

# Figure 4.8.2

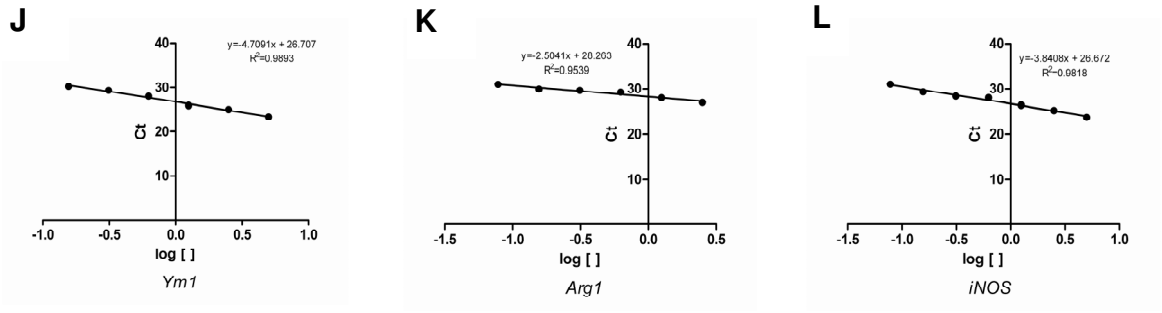

J
y=-4.7091x + 26.707
R²=0.9893
40
30
20
10
Ct
-1.0
-0.5
0.0
0.5
1.0
log [ ]
Ym1
K
y=-2.5041x + 20.203
R²=0.9539
40
30
20
10
Ct
-1.5
-1.0
-0.5
0.0
0.5
log [ ]
Arg1
L
y=-3.8408x + 25.672
R²=0.9818
40
30
20
10
Ct
-1.5
-1.0
-0.5
0.0
0.5
1.0
log [ ]
iNOS

Figure 4.8

**Alternatively activated macrophages characterise the progressive fibrotic phase of pulmonary fibrosis in mice**

Female C57Bl/6 mice were given bleomycin i.t. (50µl). Liposomal clodronate (LC) was given i.t. (100µl) on either day 21 or day 28. 0.9% saline and liposomal PBS (LPBS) were given i.t. as controls. qPCR was performed on lung homogenates (A)-(C). Immunocytochemistry (ICC) was performed on cytospins from bronchoalveolar lavage fluid (BALF) (D). Immunohistochemistry (IHC) was performed on lung sections (E)-(H). Arginase activity was performed on lung homogenates (I). (A)*Ym1* gene expression is reduced following lung macrophage depletion (bleomycin alone vs. bleomycin, B, *plus* LC on day 21, *p=0.0246). (B) *Arginase1 (Arg1)* gene expression is reduced following lung macrophage depletion (bleomycin alone vs. B *plus* LC on day 21, p=ns, trend to reduction). (C) *iNOS* gene expression is not affected following lung macrophage depletion. (D), (F) Lung macrophages from bleomycin treated mice stain for Ym1. (E) Ym1 expressing lung macrophages are reduced following lung macrophage depletion (bleomycin alone vs. B *plus* LC on day 21, p=0.071, trend to reduction; or day 28, *p=0.0233). (G)-(H) Ym1-positive lung macrophages in bleomycin treated mice are absent following lung macrophage depletion. Female C57Bl/6 mice were given 0.033mg bleomycin i.t. *plus* LC (100µl) i.t. on day 21 (G) or day 28 (H) and culled on day32. (I) Arginase activity is reduced following lung macrophage depletion (bleomycin alone vs. B *plus* LC on day 21, *p=0.0367). (J)-(L) Representative standard curves used in qPCR analysis are shown for (J) *Ym1*, bleomycin treated lung, (K) *Arg1*, bleomycin treated lung, (L) *iNOS*, bleomycin treated lung. (A)-(C) n=4-5, (D)-(H) n=6-15, (I) n=4-5. Data are presented as mean +/- SEM. Bar (D) 20µm, (F)-(H) 50µm.

**Alternatively activated lung macrophages are present in patients with IPF.**

Having suggested that alternatively activated lung macrophages are important during the progressive phase of fibrosis in a murine model of pulmonary fibrosis I wished to establish whether alternatively activated macrophages are present in human disease.

Cytospins were created from BALF from healthy volunteers and patients with IPF. The slides were stained by immunocytochemistry for the human marker of alternative macrophage activation, CD163 (Mantovani *et al*., 2002; Moestrup *et a*l., 2004; Tiemessen *et al*., 2007). As shown in Figure 4.9, A and B, CD163 is not expressed on lung macrophages from elderly healthy volunteers but is up-regulated on lung macrophages from patients with IPF, Figure 4.9, E-H. This further suggests that the alternatively activated macrophage may be important during lung fibrogenesis. Clinical details of the four patients with IPF are shown in Table 2.

# Figure 4.9

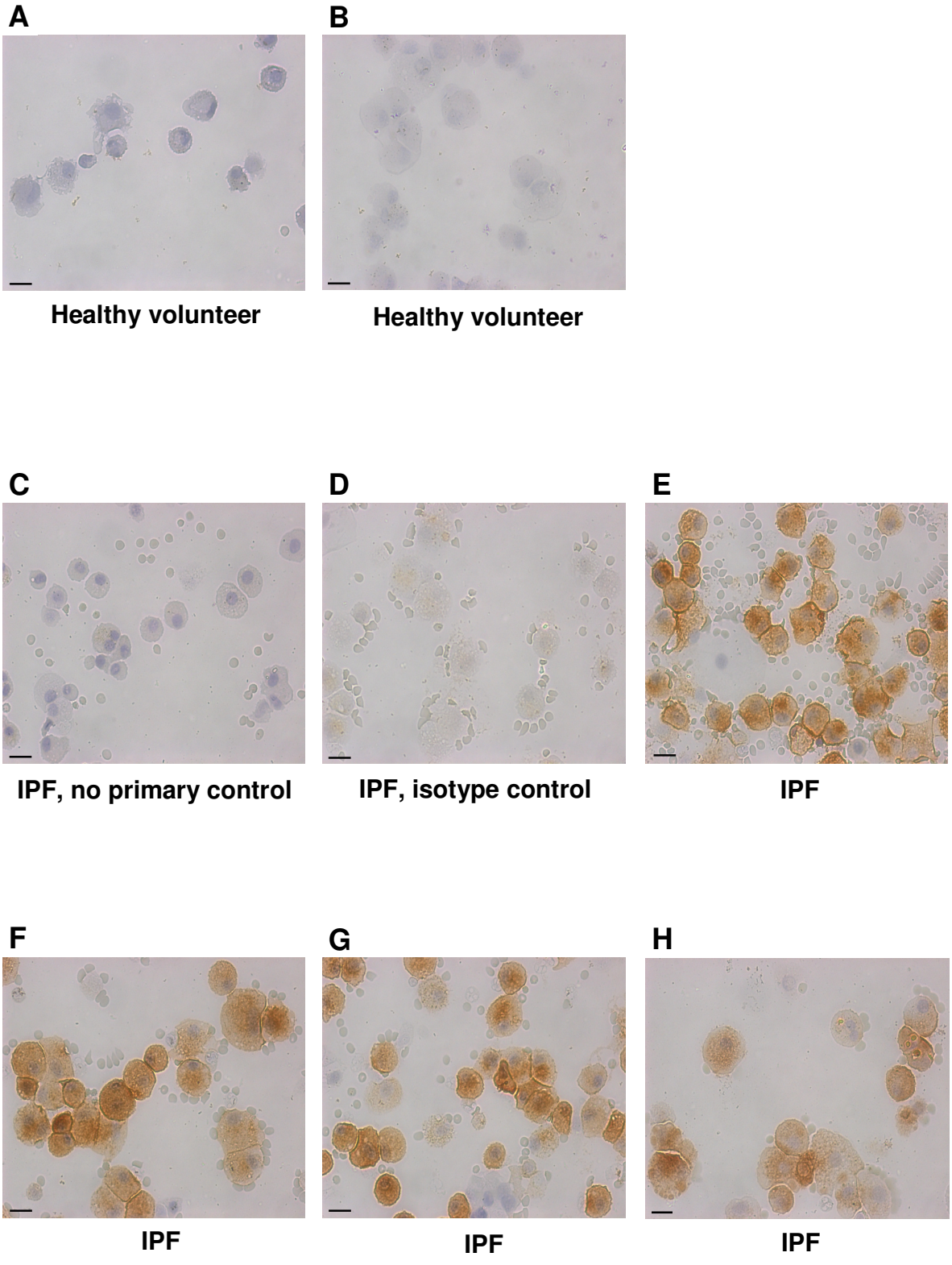

A
B
Healthy volunteer
Healthy volunteer
C
D
E
IPF, no primary control
IPF, isotype control
IPF
F
G
H
IPF
IPF
IPF

Figure 4.9

**Alternatively activated lung macrophages are present in patients with IPF.**

Cytospins were created from bronchoalveolar lavage fluid from elderly healthy volunteers (A)-(B) and patients with idiopathic pulmonary fibrosis (IPF) (C)-(H). (A)-(B) CD163 is not expressed on lung macrophages from elderly healthy volunteers. (C)-(E) Cytospins from an individual patient with IPF stained with (C) secondary antibody only, (D) isotype control and (E) CD163. (E)-(H) CD163 is expressed on lung macrophages from patients with IPF. n=2-4. Bar 20μm.

| | Patient | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Age | 65 | 50 | 79 | 77 |
| Date of 1st HRCT | 22/06/07 | 09/08/07 | 07/11/07 | 11/10/07 |
| Baseline $FEV_1$ | 1.7 (1.73)¶ | 2.79 (3.76) | 1.94 (2.13) | 2.89 (2.62) |
| Baseline VC | 2.3 (2.09) | 3.3 (4.67) | 2.38 (2.85) | 3.18 (3.48) |
| Baseline $FEV_1$/VC | 73.9% | 84.5% | 81.5% | 90.0% |
| Baseline TLco | 1.92 (6.39) | 5 (10.56) | 4.65 (6.6) | 4.06 (7.84)¶ |
| VC at 6 months | 2.25 (-2.2%)* | 2.72 (-17.6%) | 2.35 (-1.3%) | 3.3 (+3.8%) |
| TLco at 6 months | 1.86 (-3.1%) | 4.58 (-8.4%) | 4.45 (-4.3%) | 4.3 (+5.9%) |
| VC at 12 months | 2.28 (-0.9%) | 2.66 (-19.4%) | 2.2 (-7.6%) | 2.9 (-8.8%) |
| TLco at 12 months | 2.11 (+9.9%) | 4.36 (-12.8%) | 4.21 (-9.5%) | 3.35 (-17.5%)* |
| BALF date | 08/10/09 | 10/09/09 | 01/10/09 | 03/09/09 |
| BALF volumes | 3x18ml | 3x20ml | 3x18ml | - |
| BALF total cell counts | $13x10^6$ | $32x10^6$ | $17x10^6$ | $38x10^6$ |
| Cell differentials (%) | | | | |
| Macrophages | 61.5 | 75.5 | 62 | 88.5 |
| Neutrophils | 31.5 | 9.5 | 24 | 10 |
| Eosinophils | 4.5 | 3.5 | 9 | 0.5 |
| Lymphocytes | 2.5 | 11.5 | 5 | 1 |

**Table 2. Clinical details of patients with IPF.**

¶Brackets for baseline pulmonary functions tests (PFTs) are predicted values. *Brackets for subsequent PFTs are % change from baseline values.

**Intra-tracheal administration of bone-marrow derived macrophages subsequent to lung macrophage depletion does not affect fibrosis progression**

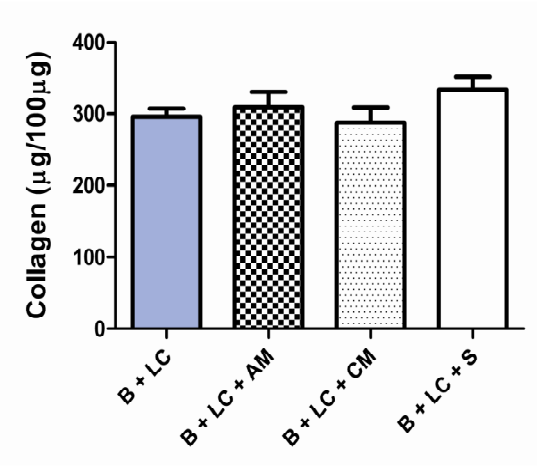
I have demonstrated that lung macrophages are required for development of fibrosis during the progressive fibrotic phase of bleomycin- and AdTGFβ- induced pulmonary fibrosis. My data also suggests that it is the alternatively activated pro-fibrotic macrophage that is responsible for this fibrosis-promoting effect. As a consequence, I aimed to establish whether intra-tracheal administration of alternatively-activated bone-marrow derived macrophages could "recover" pulmonary fibrosis subsequent to lung macrophage depletion. In addition I aimed to see what effect, if any, would be obtained if classically-activated bone-marrow derived macrophages were used instead of alternatively activated ones.

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50μl) on day 0 and liposomal clodronate (100μl) i.t. on day 21. Groups of mice were then given on day 22, either 0.9% saline (50μl) i.t., alternatively activated bone-marrow derived macrophages ($1x10^6$ in 50μl) i.t., classically activated bone-marrow derived macrophages ($1x10^6$ in 50μl) i.t., and culled on day 32. As shown in Figure 4.10A, there was no difference in fibrosis between the groups as quantified by Sircol assay. This lack of effect was corroborated by Masson's trichrome staining on lung sections for all groups as shown in Figure 4.10, B-E. The life-cycle of the macrophage is unclear. As has already been discussed, alveolar macrophages may proliferate (Tarling *et al*., 1987), or they may be derived from circulating monocytes through a lung macrophage intermediate (Landsman & Jung 2007). With this in mind it may not be surprising that a bone-marrow derived macrophage is unable to behave in the same way as a *bona fide* lung macrophage, and this would explain why this "add-back" experiment did not "recover" fibrosis. In addition, it is possible that having broken the cycle of macrophage-promoting fibrosis, addition of alternatively activated or classically activated macrophages to what is probably now a completely different molecular and cellular milieu is unable to alter the course of the disease process. Furthermore, it is unclear whether cells given intra-tracheally actually get to and engraft within the alveolar space. It is possible that the

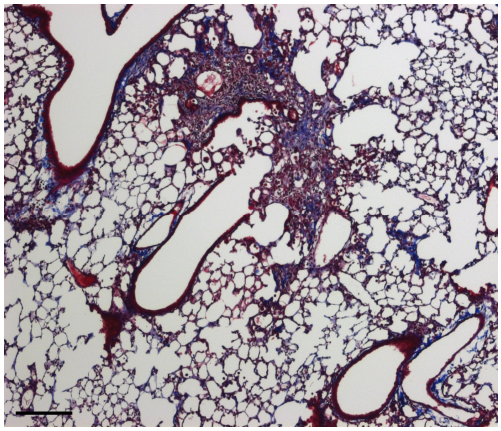
cells do not survive. A further question is whether the number of cells are sufficient. However, I believe that $1x10^6$ cells are probably within the range of cells required; this number having been chosen as a result of a previous study that showed an effect of cells "added back" by intra-tracheal route (van Rijt *et al*., 2005).

# Figure 4.10

A

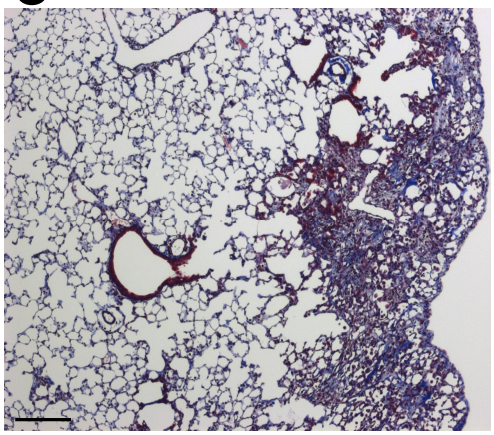



B



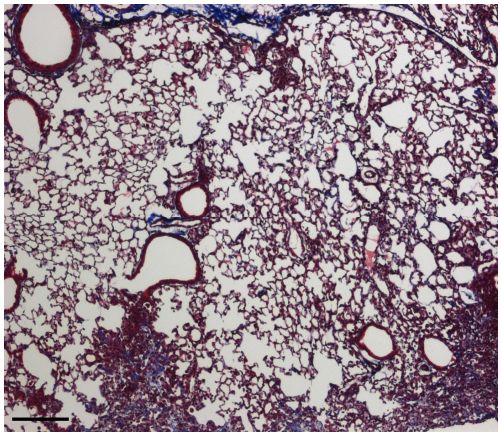
B + LC

C



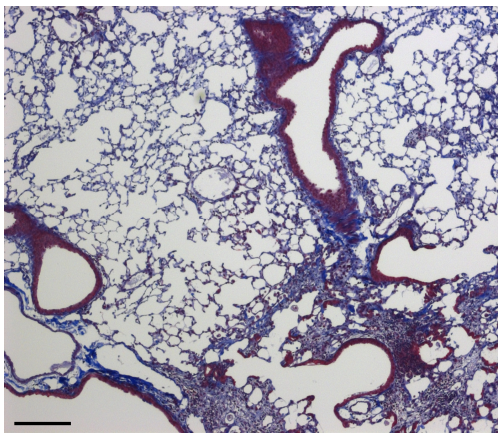
B + LC + AM

D



B + LC + CM

E



B + LC + S

Figure 4.10

**Intra-tracheal administration of bone-marrow derived macrophages subsequent to lung macrophage depletion does not affect fibrosis progression**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) on day 0 and liposomal clodronate (100µl) i.t. on day 21. In addition after a further 24 hours mice were given either 0.9% saline (50µl) i.t., alternatively activated bone-marrow derived macrophages ($1x10^6$ in 50µl i.t.) or classically activated bone-marrow derived macrophages ($1x10^6$ in 50µl i.t.). As a control, a group of mice was not given anything subsequent to i.t. liposomal clodronate. All mice were culled on day 32. Fibrosis was quantified by Sircol assay (A) and visualised by Masson's trichrome staining on lung sections (B)-(E). (A) No differences in fibrosis were detected for any of the groups when compared to the bleomycin *plus* liposomal clodronate group. This lack of effect was confirmed by Masson's trichrome staining of lung section (B)-(E), where bleomycin, B, administration was followed by i.t. administration of (B) liposomal clodronate (LC) only, (C) liposomal clodronate (LC) *plus* alternatively activated bone-marrow derived macrophages (AM) ($1x10^6$ in 50µl i.t.), (D) liposomal clodronate (LC) *plus* classically activated bone marrow-derived macrophages (CM) ($1x10^6$ in 50µl i.t.) and (E) liposomal clodronate (LC) *plus* 0.9% saline (S) (50µl i.t.). n=7-8 per group. Bar 200µm.

**Lung macrophages create a matrix-promoting microenvironment within *lung tissue***

The MMP/TIMP ratio is known to be critical for ensuring an appropriate wound healing response and for controlling lung morphostasis (Perez-Tamayo 1974). Many MMPs have been investigated in the context of both bleomycin injury and in IPF. Of particular interest are the gelatinases MMP2 and MMP9, collagenase 3 (MMP13), and their collective inhibitor TIMP1 (Swiderski *et al*., 1998; Perez-Ramos *et al*., 1999; Selman *et al*., 2000; Zuo *et al*, 2002; Atkinson & Senior 2003; Pardo & Selman 2006). Within fibrotic lung tissue the predominant matrix components are the fibrillar collagens I and III (Kirk *et al*., 1984; Raghu *et al*., 1985; Quinones & Crouch 1986).

I sought to determine the effects of alveolar macrophage depletion on *TIMP* and *MMP* gene expression and TIMP protein quantity, and specifically to establish whether any location specific differences exist. To this end I firstly examined expression and protein quantity within lung tissue.

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0 followed by liposomal clodronate (100µl) i.t. on day 21. Mice were culled on day 25. As shown in Figure 4.11, A, B and C, depletion of lung macrophages leads to a reduction in *TIMP1* and *MMP13* gene expression with no effect on *MMP2* gene expression. There is a suggestion in Figure 4.11D that the average protein quantity of TIMP1 in lung tissue, as determined by western blotting, is reduced following lung macrophage depletion as compared to non-depleted bleomycin controls. This was not however formally quantified. In addition, the GAPDH controls are not of a sufficient standard for firm conclusions to be drawn. Standard curves for the qPCR analyses are shown in Figure 4.11, E-G. Overall, these results are consistent with the possibility that macrophages may create a matrix-promoting microenvironment within *lung tissue*. Additional experiments that would have been performed, had time allowed, to add credibiltity to this assertion are western blotting for MMP13 and gelatin zymography for MMP2 (and MMP9).

# Figure 4.11

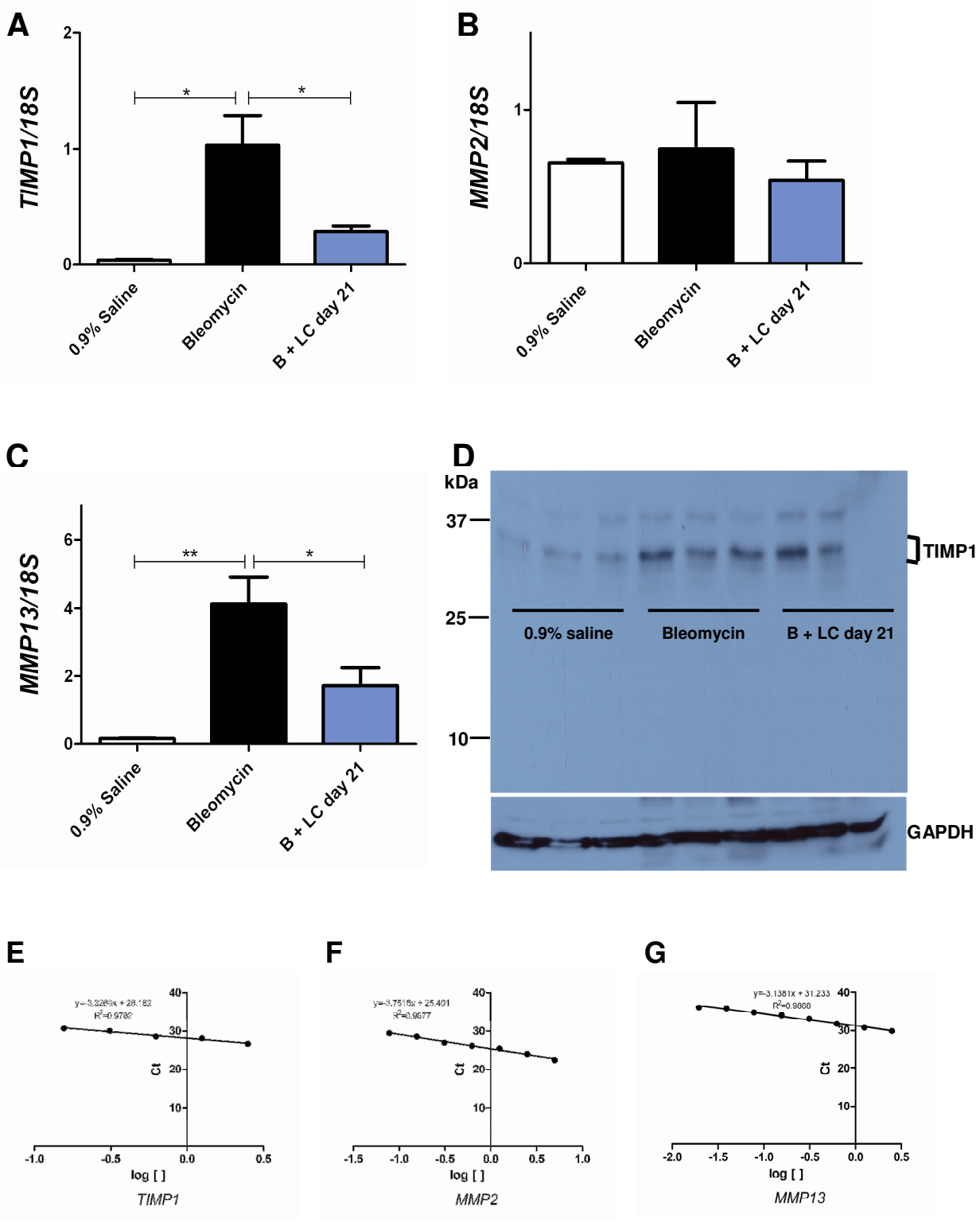

A
TIMP1/18S
2
1
0
*
*
0.9% Saline
Bleomycin
B + LC day 21
B
MMP2/18S
1
0
0.9% Saline
Bleomycin
B + LC day 21
C
MMP13/18S
6
4
2
0
**
*
0.9% Saline
Bleomycin
B + LC day 21
D
kDa
37
25
10
TIMP1
0.9% saline
Bleomycin
B + LC day 21
GAPDH
E
Ct
log [ ]
TIMP1
F
Ct
log [ ]
MMP2
G
Ct
log [ ]
MMP13

Figure 4.11

**Lung macrophages create a matrix-promoting microenvironment within *lung tissue***

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) +/- liposomal clodronate (LC) i.t. (100µl) on day 21 and culled on day 25. qPCR (A)-(C) and western blotting (D) were performed on lung homogenates . Following lung macrophage depletion there is (A) a significant reduction in *TIMP1* gene expression (bleomycin alone vs. bleomycin, B, *plus* LC on day 21, *p=0.0381), (B) no effect on *MMP2* gene expression (bleomycin alone vs. B *plus* LC on day 21, p=ns) and (C) a significant reduction in *MMP13* gene expression (bleomycin alone vs. B *plus* LC on day 21, *p=0.0485). (D) Following lung macrophage depletion there is a suggestion that the average protein quantity is reduced compared to bleomycin positive controls. Representative standard curves used in qPCR analysis are shown for (E) *TIMP1*, bleomycin treated lung, (F) *MMP2*, bleomycin treated lung, (G) *MMP13*, bleomycin treated lung. (A)-(C) n = 4-5 for each group, (D) n = 3. Results are expressed as mean +/- SEM.

**Lung macrophages create a matrix-degrading microenvironment within the *alveolar space***

Within the alveolar space the main structure composed of matrix is the basement membrane (BM). The main component of BM is type IV collagen (Nerlich 1995) which is believed to be degraded by the gelatinases MMP2 and MMP9. I wished to determine the effects of depletion of lung macrophages on *TIMP* and *MMP* gene expression within the alveolar space and to determine if there were any differences compared with the expression pattern seen in lung tissue.

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) on day 0 followed by liposomal clodronate i.t. (100µl) on day 21. Mice were culled on day 22, bronchoalveolar lavage performed, alveolar macrophages isolated, and qPCR performed on this selective cell population, or mice were culled on day 25 and lung protein quantity assessed by western blotting. As shown in Figure 4.12, A and B, depletion of lung macrophages leads to an increase in *TIMP1* expression and TIMP1 protein levels in the BALF. It is interesting to note that the increase in TIMP1, bleomycin *versus* 0.9% saline, is much more dramatic at the protein level than at the gene level. The reason for this is unclear. It is possible that TIMP1 is produced and stored, ready for secretion at an appropriate time. This could partially explain the differences; further work is required to prove this hypothesis. Moreover, formal quantification of the western blot by densitometry could confirm what is currently a visual representation rather than a firm quantifiable difference. As shown in Figure 4.12C, depletion of lung macrophages leads to a decrease in *MMP2* expression in alveolar macrophages within the alveolar space. These are opposite to the effects seen in lung tissue, and taken together suggests that location-specific differences may exist with regard to MMP/TIMP expression during the pathogenesis of lung fibrosis. It is important to state that no controls were used (such as GAPDH) in the western blot of bronchoalveolar lavage fluid (BALF). In performing western blots of lung tissue, an absolute and equivalent amount of protein (determined by BCA assay [Materials & Methods, Chapter 2, 2.17]) is added to each well. As such the western blot effectively demonstrates the amount of protein present,

previously corrected per unit weight of lung tissue, and in that setting the addition of a GAPDH control is appropriate. In BALF specimens, it is not possible to correct the protein present to the total protein in BALF. As such a GAPDH control may not be appropriate and for this reason was not used. To control the experiment as far as possible it is imperative that equal volumes of BALF are added to each well to minimise variability, and this was performed, but it must be recognised that western blots on BALF samples are inherently difficult to control, and as such caution must be used in interpreting results.

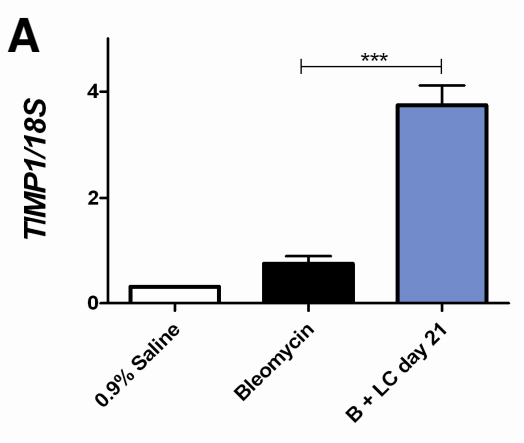
Next I established that the expression of MMP9 at day 22 following bleomycin instillation was lower than that found in the non-injured, 0.9% saline controls (Figure 4.12D). Furthermore following lung macrophage depletion, gene expression levels of *MMP9* returned to the levels seen with the 0.9% saline controls. As this was a somewhat unexpected finding I went on to perform gelatin zymography of BALF. This suggested similar findings; activity of MMP9 within the BALF appeared to decrease subsequent to bleomycin-induced pulmonary fibrosis, but returned to control levels following lung macrophage depletion (Figure 4.12E). While the overall trend for this can be seen, it is obvious that there is variability within the lanes. This limitation could have been overcome by repeating the zymogram and by using greater *n* numbers, and this would have been performed had time allowed. It has previously been shown using the bleomycin model that transgenic mice over-expressing MMP9 in alveolar macrophages have less fibrosis compared with their littermate wild-type controls (Cabrera *et al*., 2007), suggesting that MMP9 expression by alveolar macrophages in the alveolar space has a "reparative" role with respect to lung fibrosis. This finding has parallels with my apparent result as I have suggested that *MMP9* gene expression is increased during lung repair (bleomycin plus liposomal clodronate compared to bleomycin alone). Interestingly a previous study showed that MMP9 knockout mice had similar levels of fibrosis but impaired bronchiolisation compared to wild-type in response to bleomycin (Betsuyaka *et al*., 2000), again suggesting a reparative role for MMP9 expression within the alveolar space. This is contrast to MMP2 which is

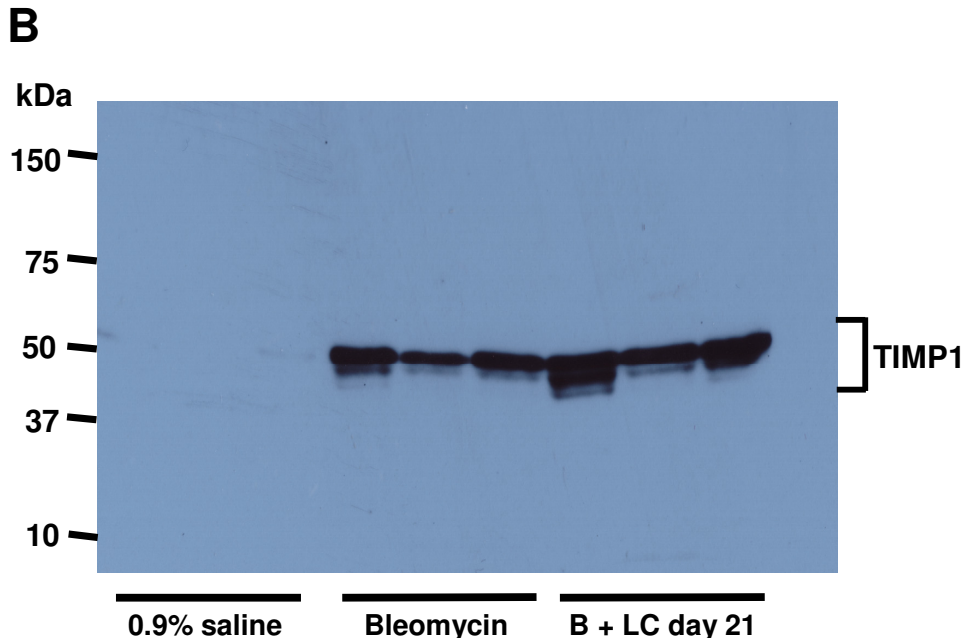
damaging to the alveolar space as a result of BM degradation. It makes logical and biological sense that the gelatinases (MMP2 and MMP9), in spite of having similar enzymatic activity, may have counter-regulatory and overlapping properties so as to allow for tissue homeostasis to be controlled at many levels, and ensure that the microenvironment may be modified appropriately in response to external stimuli.

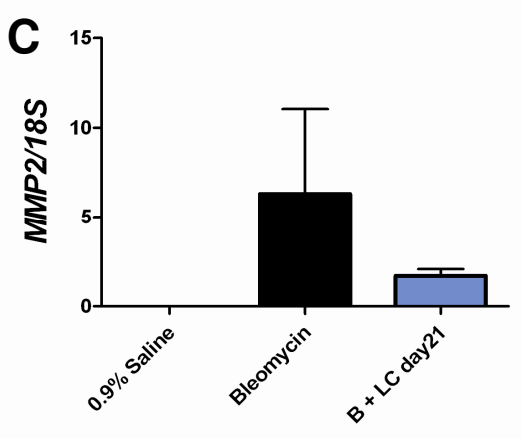
This is therefore, to my knowledge, the first demonstration of the possibility that location or compartmental specific effects of MMPs/TIMPs exist during lung fibrosis. Furthermore I suggest for the first time the possible differential effects exhibited by MMP2 and MMP9 within the alveolar space. It is clear however that considerable work will be required before clear conclusions about these possibilities can be made. However, my work has suggested that there is an ever increasing complexity to the nature of MMP/TIMP biology, and that further new insights will undoubtedly produce exciting, and possibly surprising, results.

Standard curves for the qPCR analyses are shown in Figure 4.12, F-I.

# Figure 4.12.1

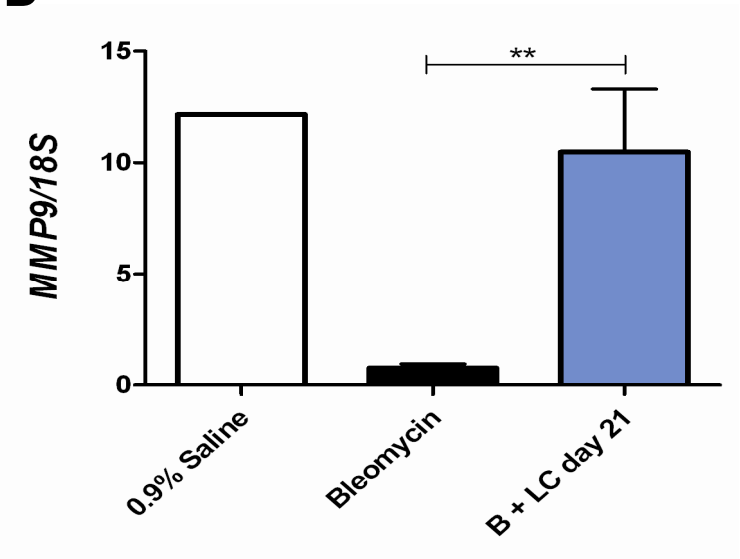

A
TIMP1/18S
***
4
2
0
0.9% Saline
Bleomycin
B + LC day 21


B
kDa
150
75
50
37
10
TIMP1
0.9% saline
Bleomycin
B + LC day 21

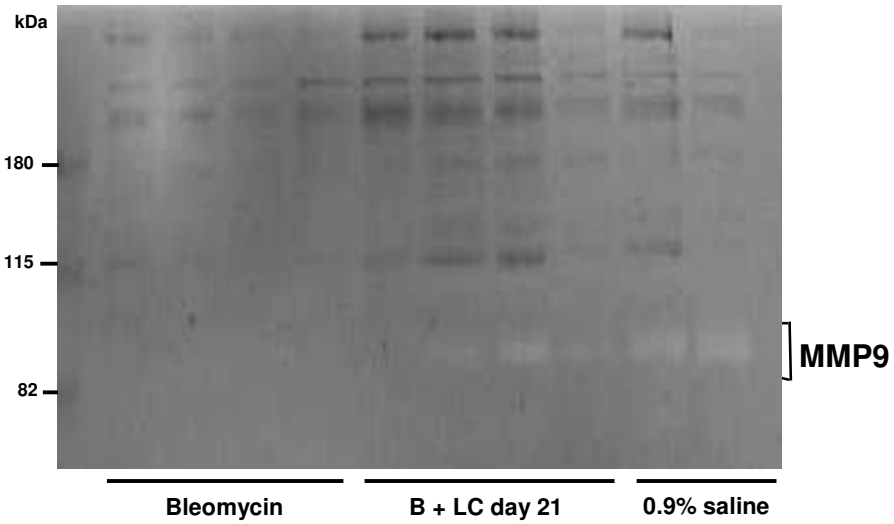

C
MMP2/18S
15
10
5
0
0.9% Saline
Bleomycin
B + LC day21

# Figure 4.12.2

D

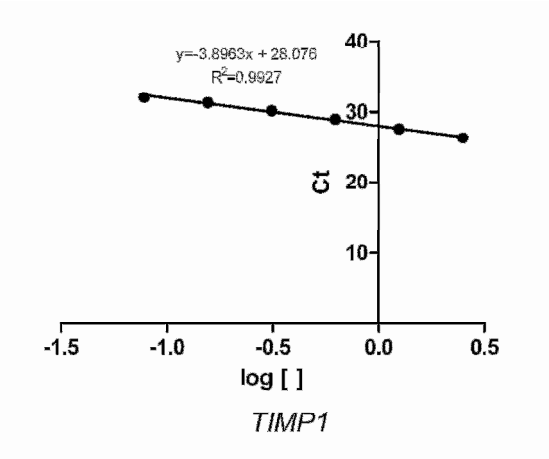



E

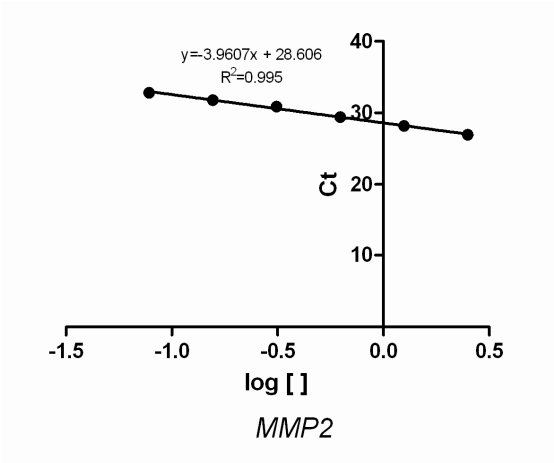


# Figure 4.12.3

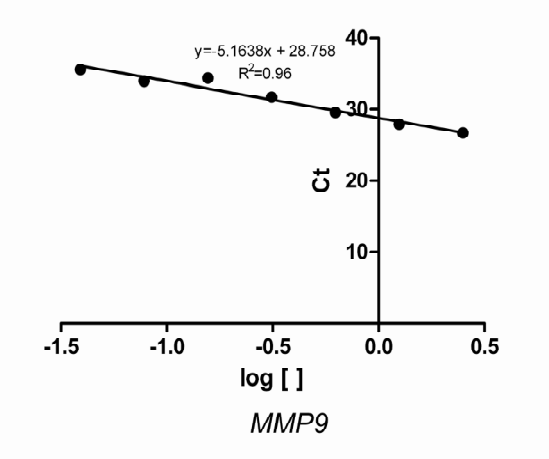
F




G




H




I

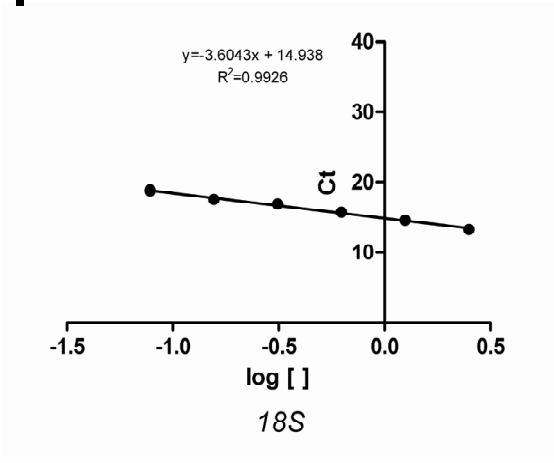


Figure 4.12

**Lung macrophages create a matrix-degrading microenvironment within the *alveolar space***

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) +/- liposomal clodronate (LC) (100µl) i.t. on day 21 and culled on day 22 (qPCR) or day 25 (western blotting, gelatin zymography). qPCR was performed on lysed alveolar macrophages (A), (C), (D). Western blotting was performed on bronchoalveolar lavage fluid (BALF) (B). Gelatin zymography was performed on BALF (E). (A) Following lung macrophage depletion there is a significant increase in *TIMP1* gene expression (bleomycin alone vs. bleomycin, B, *plus* LC on day 21, ***p<0.001). This was confirmed by western blotting (B). (C) Following lung macrophage depletion there is a trend to reduction in *MMP2* gene expression (bleomycin alone vs. B *plus* LC on day 21, p=ns, trend to reduction). (D) Following bleomycin administration there is a reduction in expression of *MMP9* by alveolar macrophages, compared to 0.9% saline controls, which is restored following lung macrophage depletion (bleomycin alone vs. B *plus* LC on day 21, **p=0.0087). (E) Following bleomycin administration there is a reduction in activity of MMP9 within the alveolar space, which returns to control levels following lung macrophage depletion. Representative standard curves used in qPCR analysis are shown for (F) *TIMP1*, BALF macrophages from bleomycin treated mice, (G) *MMP2*, BALF macrophages from bleomycin treated mice, (H) *MMP9*, BALF macrophages from bleomycin treated mice. (I) *18S*, BALF macrophages from bleomycin treated mice. (A), (C), (D) n = 5 for each group except 0.9% saline which represents a pooled sample. (D) n = 3 per group. (E) Bleomycin and B + LC day 21, n = 4; 0.9% saline, n = 2. Results are expressed as mean +/- SEM.

*4.4 Discussion*

Using a validated murine model of lung fibrosis, the bleomycin model, interrogated by two lung macrophage depletional strategies, and backing up my findings with an independent second model, I have demonstrated that lung macrophages appear to be important for lung fibrogenesis and lung fibrosis resolution.

As discussed previously, interest in the role of macrophages in lung fibrosis is not new. Several papers in the 1980s highlighted their potential importance (Rennard *et al*., 1981; Bitterman *et al*., 1983; Bitterman *et al*., 1984; Lacronique *et al*., 1984; Bitterman *et al*., 1986; Rennard *et al*., 1988; Nagaoka *et al*., 1990). However research in this area has not progressed, in part as a consequence of the lack of benefit of corticosteroids in treating fibrotic lung diseases (Turner-Warwick *et al*., 1980b). Investigation into the pathogenesis of pulmonary fibrosis has focussed on the aberrant wound healing model (Selman *et al*., 2001; Selman & Pardo 2006). Importantly, it has been demonstrated that corticosteroids actually induce alternative activation of macrophages (Gratchev *et al*., 2001; Gratchev *et al*., 2005), and a recent study has suggested that alternatively activated macrophages may regulate myofibroblasts *in vitro* (Prasse *et al*., 2006). In addition, corticosteroids have previously been shown to induce lung fibroblast proliferation (Warshamana *et al*., 1998) through up-regulation of the PDGF-α receptor. I have discussed previously that PDGF is up-regulated in alveolar macrophages of patients with IPF (Nagaoka *et al*., 1990), as mentioned above alternatively activated macrophages regulate myofibroblasts *in vitro* (Prasse *et al*., 2006). Several other papers have suggested the potential importance of macrophages (Mora *et al*., 2006; Prasse *et al*., 2006; Okuma *et al*., 2004). My study is the first to thoroughly investigate the role of macrophages in pulmonary fibrosis.

Previous studies have attempted to ascertain the role of macrophages in lung fibrosis but they have all used models that have methodological concern (Zhang-Hoover *et al*., 2000; Misson *et al*., 2004; Bem *et al*., 2008). The study by Bem and colleagues (Bem *et al*.,

2008) looked at the effect of depleting lung macrophages, also using liposomal clodronate, during a lung injury protocol. This study showed that depletion of lung macrophages, prior to the initiation of lung injury protocol, did not prevent Fas-mediated lung injury in mice. The Fas-mediated lung injury model has been used as a model of lung fibrosis (Matute-Bello *et al*., 2007). It is produced by administration of the Fas-activating antibody Jo2, which induces apoptosis of alveolar epithelial cells and consequent fibrosis. Importantly, fibrosis only occurs after long-term administration of Jo2 (Matute-Bello *et al*., 2007). Short term administration induces lung injury characterised by inflammation, not fibrosis (Matute-Bello *et al*., 2007). The study by Bem and colleagues used short-term administration and therefore is not a model of lung fibrosis *per se*. They showed that depletion of resident lung macrophages pre-administration of the Jo2 antibody had no effect on lung injury as shown by lung histology. In fact their histological analysis of lungs actually showed a worsening of lung injury. Furthermore, it is questionable whether depletion of lung macrophages pre-administration of the fibrosis-inducing agent has any relevance to the role of lung macrophages during injury. The study by Zhang-Hoover and colleagues (Zhang-Hoover *et al*., 2000) analysed the effect of depleting lung macrophages in a model of hapten immune pulmonary interstitial fibrosis induced by a recall cell-mediated immune response against the hapten 2,4,6-trinitrobenzene sulphonic acid (TNBS) in the lung. This model has not been well characterised, and it is questionable whether it is a model of lung fibrosis *per se* and as such no comparisons can be made with my own study nor with IPF. The study by Misson and colleagues (Misson *et al*., 2004) did not find any relationship between macrophage activation state and the promotion of lung fibrosis. However, in their study they used silica to induce lung fibrosis. It is known that silica is toxic to macrophages, so any effect that macrophages may have during lung fibrogenesis would have been confounded by their model itself. Furthermore, the granulomatous lesions produced by silica have a strong lymphocyte and mast cell component to their response, which probably has a strong immunogenic component, and as such limits the usefulness of silica as a model of lung fibrosis, particularly with regard to the investigation of macrophages.

Recent work by many groups has shown the heterogeneity that macrophages exhibit, culminating in an attempt to categorise macrophages into subtypes, or activation states, according to protein and RNA expression patterns, function, and the stimuli required to induce these activation states (Wynn *et al*., 2004; Gordon & Taylor 2005). Borne out of this classification are alternatively activated macrophages. As discussed previously, they have anti-inflammatory properties, but it is suggested that their major effects are likely to be in the control of wound healing and repair, and as a result alternatively activated macrophages may stimulate extra-cellular remodeling and fibrogenesis (Gordon 2003; Varin & Gordon 2009).

I have shown that lung macrophages do not appear to be involved in the early inflammatory phase of bleomycin-induced lung fibrosis, but show using two independent macrophage depletional strategies that they are important during the subsequent progressive fibrotic phase. Moreover I demonstrate the importance of lung macrophages in the adenoviral-TGFβ-mediated model of progressive lung fibrosis. TGFβ is widely recognized to be a "master" molecule during the development of lung fibrosis (Munger *et al*., 1999; Sheppard 2006) and the fact that lung macrophage modulation limits induced lung fibrosis emphasises their importance. It is likely that by using the CD11c-DTR mouse there will be an inherent depletion of lung dendritic cells as well as lung macrophages. However, it has been demonstrated that lung macrophages are the predominant $CD11c^+$ cell type in the lung and within the alveolar space (Landsman & Jung 2007), and it has been shown that DT depletes interstitial and alveolar macrophages (Landsman & Jung 2007). Furthermore, and as has already been discussed, the distinctions between macrophages and dendritic cells are becomingly increasingly blurred (Hume 2008)

For the first time, I have assessed the importance of lung macrophages during the resolving phase of bleomycin-induced lung fibrosis; it is compelling that lung macrophages are required for the resolution of fibrosis. This is in keeping with recent work which has shown that alveolar macrophages are involved in the removal of

accumulated collagen (Atabai *et al*., 2009). This result is novel in two ways: I am not aware of any publications looking at the biology of the resolution phase of bleomycin-induced pulmonary fibrosis, and secondly, I have not only demonstrated for the first time the important role that lung macrophages play in the progression of pulmonary fibrosis, I have also shown that they exhibit distinct and opposing effects during the resolution of pulmonary fibrosis. This data is in keeping with the findings in experimental liver fibrosis where it has been established that macrophages promote liver fibrogenesis, but in contrast they also facilitate liver fibrosis resolution (Duffield *et al*., 2005).

My study demonstrates the novel finding that i.t. instillation of bleomycin induces up-regulation of the alternative macrophage markers Ym1 and arginase1, and that following lung macrophage depletion, gene expression and protein levels are reduced for both of these markers. Ym1 is an archetypal murine marker of alternatively activated macrophages (Raes *et al*., 2002). The absolute function of Ym1, also called chitinase 3-like protein 3, is uncertain. Recent work, however, has suggested that it is a marker of oxidative stress, known to be important in the pathogenesis of lung fibrosis (Walters *et al*., 2008). No human homologue has been identified as yet; however, YKL-40 (also called chitinase 3-like protein 1) is a member of the chitinase family of proteins. Circulating levels (Johansen *et al*., 1997; Johansen *et al*., 2000; Zheng *et al*., 2005) and liver tissue staining (Johansen *et al*., 2000) of YKL-40 have been found to be elevated in patients with liver fibrosis and it has recently been proposed that it has a role in tissue remodeling and fibrosis (Lee & Elias 2010). Arginase1 is another characteristic marker of alternative macrophage activation. It is necessary for the conversion of L-arginine to L-ornithine. L-ornithine is subsequently converted to proline which is required for the formation of collagen (Gordon 2003). As such arginase1 has been implicated in wound healing, repair and fibrogenesis. Importantly arginase1 is known to be up-regulated in patients with IPF (Mora *et al*., 2006).

I have demonstrated that alternatively activated lung macrophages are present in patients with IPF. This lends support to my suggestion that the alternatively activated macrophage phenotype is important for lung fibrogenesis. It is important to note some limitations of this work. I have shown that depletion of macrophages leads to amelioration of lung fibrosis, and that this is associated with a reduction in alternative macrophage markers. I have not shown directly that alternatively activated macrophages are depleted *per se*. Furthermore, I have not looked specifically at the quantitites or proportions of macrophages subtypes during lung fibrogenesis, and as a result have not categorically delineated the overriding macrophage phenotype present during lung fibrogenesis. It may be argued that if macrophages are effectively depleted, and a major resultant effect is reduction in alternative macrophage markers (as opposed to classically activated macrophage markers), that alternatively activated macrophages are most likely the predominant macrophage subtype; however, absolute confirmation of this would undoubtedly add extra credibility to my assertions. A further limitation is that this work has not taken specific account of the other cell types present and their possible effects. However, the specific aims of this thesis were to assess the role of macrophages during lung fibrogenesis, which I believe I have gone some way to addressing. The role of other "immune-type" cells is obviously of paramount importance, and requires proper investigation, but was not able to be properly assessed within the limitations of this thesis. What is important to say, particularly in the context of bleomycin-induced lung fibrosis, is that monocytes and macrophages become the predominant infiltrating cell as the disease progresses through to the fibrotic stage (Izbicki *et al*., 2002; Higashiyama *et al*., 2007).

The potential importance of location-specific functions of matrix metalloproteinases (MMPs) and their inhibitor, tissue inhibitor of matrix metalloproteinases (TIMP1) has recently been highlighted (Pardo *et al.*, 2008). TIMP1 is known to be up-regulated in bleomycin induced pulmonary fibrosis (Swiderski *et al.,* 1998; Madtes *et al*., 2001); a finding replicated by myself. I sought to identify whether lung macrophages promote lung fibrosis by mechanisms that rely on compartmental specific expression and

function of MMPs and their inhibitor. I demonstrate that this appears to be true, as depletion of lung macrophages results in a reduction in *TIMP1* gene expression within lung tissue, but an increase (in gene expression and protein) in the alveolar space. MMP2 is primarily active against type IV collagen, the major constituent of basement membrane (BM), and has a weak ability to degrade interstitial fibrillar collagens (types I and III) (Aimes *et al*., 1995; Patterson *et al*., 2001). It is up-regulated early in bleomycin induced pulmonary fibrosis and it has been postulated that MMP2 production may disrupt the BM and aid in the progression of bleomycin induced pulmonary fibrosis (Swiderski *et al*., 1998) and IPF (Pardo & Selman 2006). I found no change in *MMP2* gene expression following lung macrophage depletion in lung tissue, albeit at a later time-point than that evaluated by Swiderski and colleagues. However, as *TIMP1* gene expression was reduced, the MMP/TIMP1 balance favoured matrix degradation following lung macrophage depletion. However, within the alveolar space, where MMP2 possibly mediates its major effects during lung fibrosis due to its predilection for collagen type IV, I found a reduction in *MMP2* gene expression, findings in keeping with the hypothesis that MMPs and TIMP1 have location-specific functions during pulmonary fibrosis. MMP13 (collagenase 3) has a similar structure and enzymatic properties to MMP1 (collagenase 1, human collagenase) but has been shown, like TIMP1, to be up-regulated in fibroblasts in response to TGFβ (Uria *et al*., 1998). Furthermore, MMP13 has been shown to be up-regulated in a murine model of lung fibrosis induced by asbestosis (Shukla *et al*., 2006) and in models of liver fibrosis (Uchinami *et al*., 2006; Fallowfield *et al*., 2007). I show that *MMP13* gene expression is reduced following lung macrophage depletion, in keeping with lung macrophages producing a matrix-promoting micro-environment within lung tissue during lung fibrosis. The role of MMP9 in pulmonary fibrosis is more complicated. Studies have shown that *MMP9* gene expression is up-regulated in tissues of patients with IPF (Zuo *et al*., 2002). Furthermore immunoreactive studies have shown that MMP9 is active on alveolar, but not interstitial macrophages (Selman *et al*., 2000). This combined with the fact that MMP9 has activity against collagen type IV, the major component of BM, lead investigators to speculate that MMP9 is involved in the pathogenesis of IPF through its

ability to degrade BM. However, MMP9 knockout mice do not exhibit reduced fibrosis in response to bleomycin, but instead exhibit impaired bronchiolisation (Betsuyaku *et al*., 2000). Alveolar bronchiolisation is the appearance of terminal airway-like epithelial cells in areas of injured alveoli. This phenomenon can be seen in response to many types of alveolar injury, and is believed to be a sign of terminal airway epithelial cell migration to these areas of injury and not a defect in alveolar epithelial cell differentiation. With this in mind it has been proposed that MMP9 plays a role in epithelial repair and not in alveolar fibrosis (Atkinson & Senior 2003). Indeed a recent study in which transgenic mice over-expressing MMP9 in alveolar macrophages were exposed to bleomycin showed that these mice had less fibrosis compared to wild-type (Cabrera *et al*., 2007), supporting a potential role for alveolar-macrophage-produced-MMP9 in facilitating BM repair, rather than degradation. Furthermore, in patients with cryptogenic organising pneumonia (COP), a condition with considerable reversibility, the MMP9/TIMP1 ratio in BALF is considerably higher than in patients with IPF (Choi *et al*., 2002). I show that gene expression of *MMP9* in alveolar macrophages is reduced in the late phase of bleomycin-induced pulmonary fibrosis, but returns to baseline levels following liposomal clodronate administration. This effect was confirmed by gelatin zymography for MMP9, and adds support to the proposal that MMP9 plays a protective rather than injurious role within the alveolar space in response to fibrotic lung injury. Taken together, I have shown for the first time the opposing effects that gelatinases may exhibit within the alveolar space in response to fibrotic lung injury. These novel findings highlight the increasing complexity of control and function of MMPs/TIMPs in lung tissue and within the alveolar space in response to fibrotic lung injury, and may require that we re-evaluate our understanding of MMP and TIMP biology.

Aside from the effects on MMP/TIMP biology, it is tempting and important to speculate on other mechanisms by which macrophages may promote fibrogenesis and facilitate lung fibrosis resolution. During lung fibrogenesis I anticipate that macrophages release soluble factors that are important for both myofibroblast survival and epithelial activation. The paper by Prasse and colleagues demonstrated that culture supernatants

of alveolar macrophages from patients with IPF increased collagen production by normal lung fibroblasts partly mediated via CCL18, a marker of alternative macrophage activation (Prasse *et al*., 2006).

Another candidate soluble factor is IGF-1. Secretion of IGF-1 by alternatively activated macrophages protects myofibroblasts from apoptosis (Wynes *et al*., 2004). It is well established that both lung tissue and BAL levels of IGF-1 are increased in patients with IPF, as well as bleomycin-induced pulmonary fibrosis (Rom *et al*., 1988; Homma *et al*., 1995; Maeda *et al*., 1996; Moestrup & Møller 2004). In a study of lung biopsy samples from patients with IPF it was found that there was a correlation between IGF-1 expression by interstitial lung macrophages and parameters of disease severity (Uh *et al*., 1998). Furthermore antibody blockade of the murine IGF-1 receptor during the inflammatory and fibrotic phases, or initiated part-way through and onwards from the inflammatory phase of the bleomycin-induced lung fibrosis model improves outcome and decreases fibrosis (Choi et al., 2009).

The importance of TGFβ as a master regulator of fibrosis is well described (Sheppard 2006). It has been demonstrated that alveolar macrophages from patients with IPF, but not from normal patients, secrete active TGFβ and are likely to be its major source (Khalil *et al*., 1989). In addition it has been proposed that TGFβ may induce epithelial to mesenchymal transition in patients with IPF (Willis *et al*., 2005). Interestingly a study of patients with IPF using *in situ* hybridisation demonstrated that $TGF\beta_1$ mRNA was present in macrophages adjacent to fibroblastic foci, but that the foci themselves showed no signal for $TGF\beta_1$ mRNA (Broekelmann *et al*., 1991).

I would speculate therefore that in patients with IPF the initial insult results from interactions between not only epithelia and fibroblasts, but through a much more complicated and concerted interplay between macrophages, epithelium and fibroblasts/myofibroblasts. I propose that once the fibroblastic focus has been set up as

the driver of matrix production, the macrophages continue to surround and feed this cellular hotspot with a variety of soluble mediators and engage in cell-cell interaction.

In summary therefore I have demonstrated for the first time *in vivo* that alternatively activated lung macrophages are required for lung fibrogenesis in a murine model of lung fibrosis and are present in the human form of the disease. I do not believe my findings detract from prevailing hypotheses regarding the pathogenesis of pulmonary fibrosis but believe they add a further layer of complexity. My finding of decreased resolution of pulmonary fibrosis subsequent to lung macrophage depletion is an intriguing one. I believe it will open up a new dimension in lung fibrosis biology and suggests a potential therapeutic strategy of macrophage-mediated fibrosis resolution. Further work will need to be performed to complete the knowledge of the role of lung macrophages in fibrotic lung disease, both during progression and resolution. New insights into pathogenesis will allow new approaches to be adopted, enabling new therapies to be produced. With the rapidly increasing research into monocyte/macrophage based cell therapy, therapeutic manipulation of the activation state of alveolar and interstitial macrophages may be contemplated to alter the course of pulmonary, as well as other fibrotic diseases.

# CHAPTER 5

## CIRCULATING MONOCYTE REGULATION OF PULMONARY FIBROSIS

*5.1 Abstract*

As there has been much controversy regarding the role of lung macrophages in pulmonary fibrosis it is no surprise that there has been little interest in the role of circulating monocytes, the precursors of tissue macrophages, in the pathogenesis of pulmonary fibrosis. Using *in vivo* depletional strategies and adoptive transfer techniques I have demonstrated for the first time the important role that circulating monocytes have in the development of pulmonary fibrosis. I have identified the Ly6C$^{hi}$ monocyte as the subtype responsible for the fibrosis-promoting effect. Recent work suggests that these cells may have an "alternatively activated" phenotype, and in keeping with this I have demonstrated that Ly6C$^{hi}$ monocyte depletion leads to a reduction in gene and protein expression of markers of alternative activation within lung tissue. By defining a new cell type of significant importance to the development of pulmonary fibrosis, I envisage that this work will enable new targets to be produced and hopefully allow for effective agents to be developed in the battle to treat pulmonary fibrosis.

*5.2 Introduction*

Pulmonary fibrosis has already been described as a devastating disorder for which there are no effective therapies. The only treatment option that has been cited as life-prolonging is lung transplantation, but as the majority of patients are over 60 by the time that they develop this lung condition then it is not surprising that very few patients with pulmonary fibrosis actually get assessed for lung transplant. In addition 50% patients on the transplant list are still waiting for a transplant 1 year after being placed on the list (Transplant activity in the UK 2007-2008) and as the median survival of IPF is approximately 3 years (Bjoraker *et al*., 1998) then it is not surprising that patients with IPF have the highest rate of death amongst patients awaiting a lung transplant (Hosenpud *et al*., 1998). In fact, as a means of trying to avert this occurrence, patients in the USA with IPF are given a three month waiting advantage over patients with emphysema (Dark 1998). As such it is imperative that attempts are made to better understand the natural history and pathogenesis of pulmonary fibrosis to allow new therapies to be developed.

With the advent of cell therapy, it has become appealing to attempt to identify cell types involved in the development of disease with the ultimate aim of manipulating cellular function to alter disease outcomes. In this thesis I have hypothesised that lung macrophages play a role in the promotion of pulmonary fibrosis. As bone-marrow and circulating monocytes are believed to be the precursors of tissue macrophages (Hunter *et al*., 2009), then it would seem logical to attempt to define the monocytes that may be the precursors of these tissue fibrosis-promoting macrophages.

In the last few years there has been an expansion in the knowledge of blood monocyte origin and differentiation. Currently there are believed to be two major types of monocytes in both man and mouse. In man the two main subtypes are $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$. These are believed to bare resemblance to the mouse subtypes $Ly6^{lo}$ and $Ly6C^{hi}$, respectively (Geissmann *et al*., 2003). It has long been suggested that there is a

monocyte subset termed the "inflammatory" monocyte (van Furth *et al.,* 1973), and it is becoming increasingly clear that the Ly6C$^{hi}$ marker defines this population of cells (Gordon & Taylor 2005).

I set out to establish whether depletion of circulating monocytes would have any effect on pulmonary fibrosis as illustrated using the bleomycin and AdTGFβ models. I demonstrate that depletion of circulating monocytes leads to a reduction in pulmonary fibrosis in both these models. I establish that it is the Ly6C$^{hi}$ population of cells that is depleted in experiments where there is a reduction in fibrosis, and show that adoptive transfer of these cells leads to a worsening of fibrosis using the bleomycin model. Finally, I demonstrate that depletion of circulating monocytes leads to a reduction in the expression of markers of alternatively activated macrophages within the lung tissue, and as such suggest that Ly6C$^{hi}$ monocytes may represent the precursors of lung tissue alternatively activated macrophages.

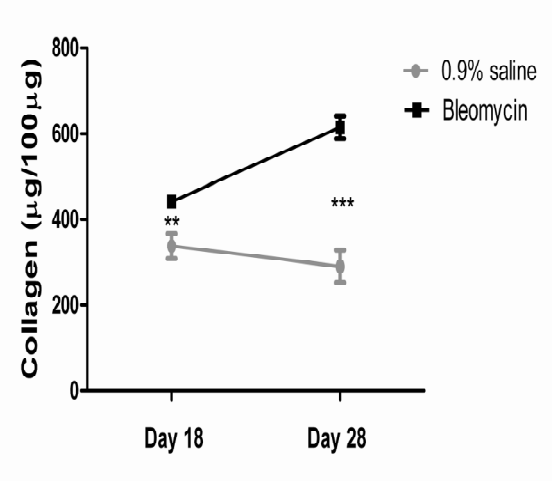
*5.3 Results*

**Bleomycin time course**

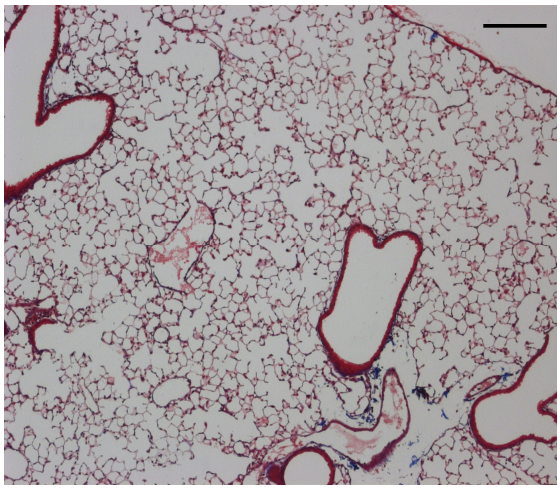
I have already demonstrated in chapters 3 and 4 that fibrosis peaks around 28 (to 32) days in the bleomycin model and around 14 days in the AdTGFβ model. In addition I have shown that at 18 days there is early fibrosis. These findings are summarised in Figure 5.1, A-D. Interestingly, there is a significant increase in peri-vascular collagen deposition at 28 days in mice treated with bleomycin, compared to mice treated with bleomycin and culled at 18 days, or mice treated with 0.9% saline (Figure 5.1, D, C and B, respectively), which in itself as an observation suggests that the circulation may be a source of factors important for the development of pulmonary fibrosis.

# Figure 5.1

A

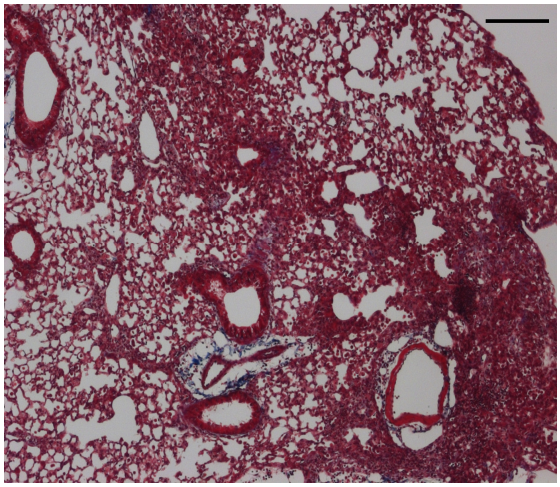



B



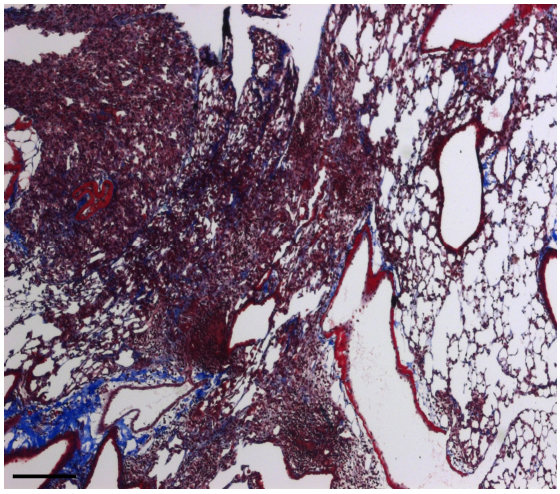
0.9% saline

C



Bleomycin day 18

D



Bleomycin day 28

Figure 5.1

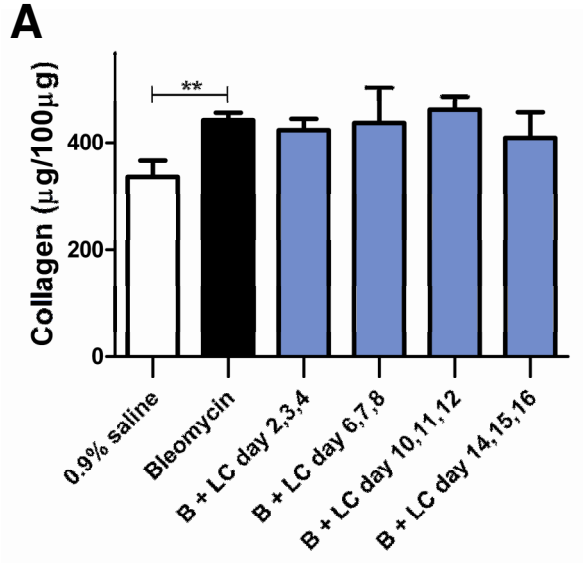
**Bleomycin time-course**

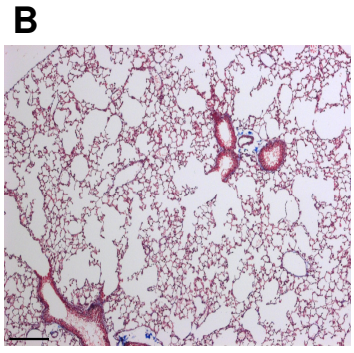
Female C57Bl/6 mice were given 0.033mg bleomycin intra-tracheally (i.t., 50µl) and culled on day 18 or 28. 0.9% saline was given i.t. (50µl) as a control. Fibrosis was measured by Sircol assay (A), or visualised by Masson's trichrome staining of representative lung sections (B)-(D). (A) Mice were given 0.9% saline or bleomycin by i.t. instillation, culled during early lung fibrosis (day 18) or progressive fibrosis (day 28) and lung collagen assessed. Significant differences in lung collagen were found between 0.9% saline-treated and bleomycin-treated groups at both 18 days (**, $p=0.0051$) and 28 days (***, $p<0.001$). (B) Masson's trichrome stain, 0.9% saline-treated mice, culled on day 18. (C) Masson's trichrome stain, bleomycin-treated mice, culled on day 18. (D) Masson's trichrome stain, bleomycin-treated mice, culled on day 28. Bar 200µm.

**Circulating monocytes are not necessary for the inflammation initiation of fibrosis**

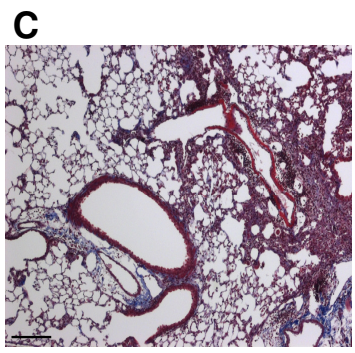
To assess the role of circulating monocytes during the inflammatory phase of bleomycin-induced lung fibrosis, I administered a single i.t. instillation of 0.033mg bleomycin and culled mice after 18 days. Liposomal clodronate (400µl) was given intra-peritoneally (i.p.) to deplete circulating monocytes on three consecutive days, either days 2, 3, 4 or 6, 7, 8 or 10, 11, 12 or 14, 15, 16. As controls, bleomycin alone or 0.9% saline (50µl) were given i.t. on day 0 and mice culled on day 18. As shown in Figure 5.2A, depletion of circulating monocytes has no effect on the inflammatory phase and early induction of lung fibrosis as measured by Sircol assay. This lack of effect was confirmed by Masson's trichrome staining on lung sections as can be seen in representative images shown in Figure 5.2, B-G.

# Figure 5.2

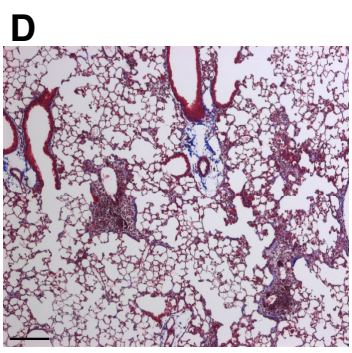


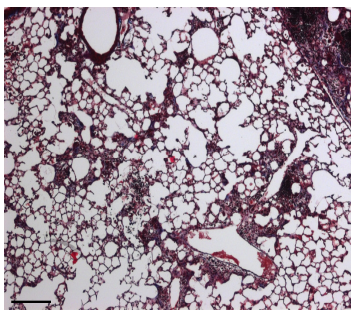

0.9% saline



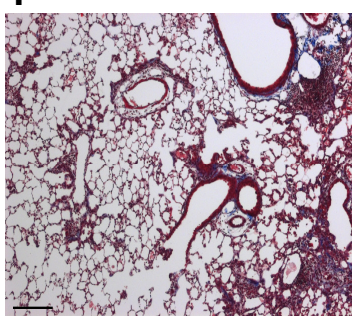

Bleomycin



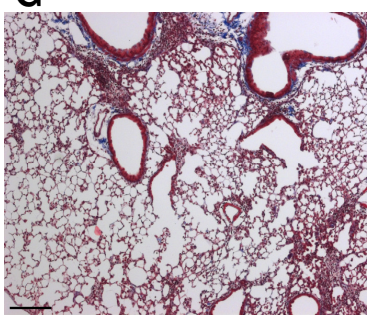

B + LC day 2,3,4




B + LC day 6,7,8




B + LC day 10,11,12




B + LC day 14,15,16

Figure 5.2

**Circulating monocytes are not necessary for the inflammation initiation of fibrosis**

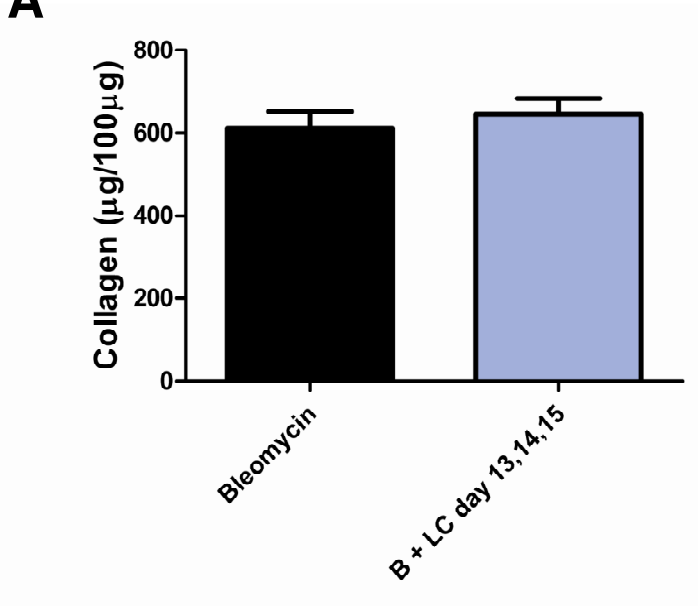
Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 18. In addition to bleomycin, groups of mice were also given liposomal clodronate by intra-peritoneal injection (i.p.) (400µl) on three consecutive days at various stages post-bleomycin instillation. All mice were culled on day 18. Fibrosis was measured by Sircol assay. 0.9% saline (50µl) was given i.t. as a control and mice culled on day 18. (A) A significant difference in fibrosis was found between bleomycin and control (0.9% saline vs. bleomycin, **p=0.0051), but there were no differences, compared with bleomycin positive control (B), when liposomal clodronate (LC) was given in combination with bleomycin. (B)-(G) Masson's trichrome staining on representative lung sections of mice treated with: (B) 0.9% saline alone, (C) Bleomycin alone, (D) Bleomycin *plus* LC i.p. on days 2,3,4. (E) Bleomycin *plus* LC i.p. on days 6,7,8. (F) Bleomycin *plus* LC i.p. on days 10,11,12. (G) Bleomycin *plus* LC i.p. on days 14,15,16. n=6-8 per group. Data are presented as mean +/- SEM. Bar 200µm.

**Depletion of circulating monocytes during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on subsequent pulmonary fibrosis**

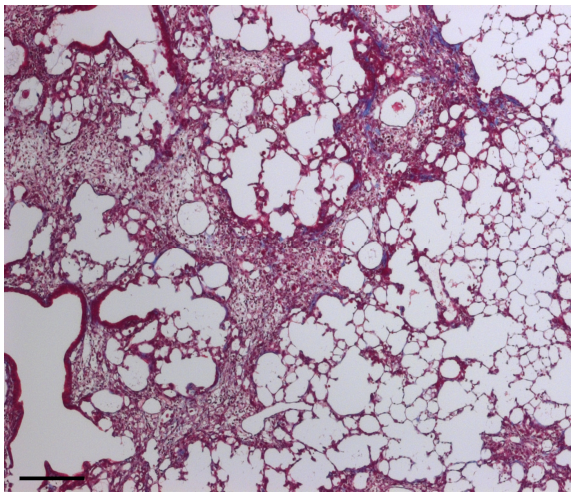
To assess whether circulating monocytes present during the inflammatory phase of bleomycin-induced lung fibrosis play in a role in the subsequent development of pulmonary fibrosis, I administered a single i.t. instillation of 0.033mg bleomycin and culled mice after 28 days. Liposomal clodronate (400µl) was given intra-peritoneally (i.p.) on three consecutive days, 13, 14 and 15. As shown in Figure 5.3A, depletion of circulating monocytes during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on the subsequent development of pulmonary fibrosis, as measured by Sircol assay. This lack of effect was confirmed by Masson's trichrome staining on lung sections as can be seen in representative images shown in Figure 5.3, B and C.

# Figure 5.3

A

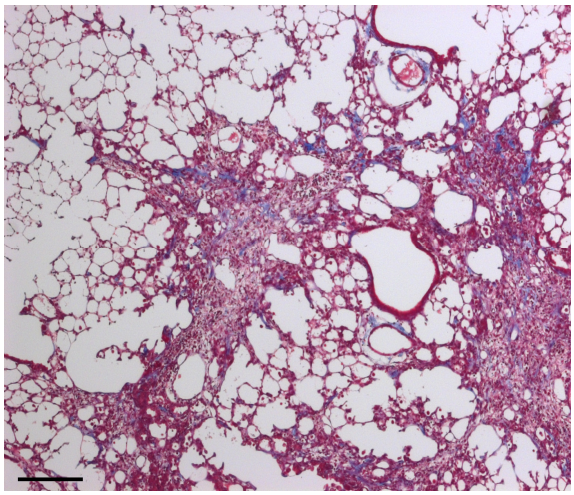



B



Bleomycin

C



B + LC day 13,14,15

Figure 5.3

**Depletion of circulating monocytes during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on subsequent pulmonary fibrosis**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl). In addition to bleomycin, a group of mice were also given liposomal clodronate (LC) intra-peritoneally (i.p.) (400µl) on three consecutive days: 13, 14 and 15. All mice were culled on day 28. Fibrosis was measured by Sircol assay. (A) No difference in fibrosis was found following circulating monocyte depletion (bleomycin vs. bleomycin, B, *plus* LC on days 13, 14, 15, p=ns). (B) and (C) Masson's trichrome staining on representative lung sections of mice treated with: (B) bleomycin alone or (C) bleomycin *plus* LC i.p. on days 13, 14, 15. n=4-5 per group. Data are presented as mean +/- SEM. Bar 200µm.

**Liposomal clodronate preferentially depletes "inflammatory" circulating monocytes**

Administration of liposomal clodronate is a well established method of selectively and effectively depleting circulating monocytes (van Rooijen & Sanders 1994; Getts *et al*., 2008; Jaiswal *et al*., 2009). Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl), liposomal clodronate by intra-peritoneal (i.p.) injection (400µl) on days 21, 22 and 23, and culled on day 24 or day 28. Control groups of mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 24 or day 28. Using flow cytometric analysis of mouse whole blood I have demonstrated that 24 hours after the final injection of liposomal clodronate there is depletion of circulating monocytes, as defined by dual CD11b and CD115 positivity (Figure 5.4, A, B and C). After a further 96 hours levels of circulating monocytes are returning to control levels (Figure 5.4, A, D and E).

As discussed in the introduction, populations of mouse monocytes have recently been subdivided into two major subgroups, $Ly6C^{lo}$ and $Ly6C^{hi}$ (Gordon & Taylor, 2005). The $Ly6C^{lo}$ group has been further classified as $Ly6C^{lo}$, $CX3CR1^{hi}$, $CCR2^{-}$, $7/4^{mid}$. They may play a role in patrolling non-inflamed tissues and differentiate into tissue-resident macrophages (Geissmann *et al*., 2003; Taylor *et al*., 2003; Auffray *et al*., 2007; Westcott *et al*., 2009). The $Ly6C^{hi}$ group has been further classified as $Ly6C^{hi}$, $CX3CR1^{lo}$, $CCR2^{+}$, $7/4^{hi}$. They are also termed "inflammatory" monocytes as they appear to be preferentially recruited to sites of tissue inflammation (Taylor *et al*., 2003; Gordon & Taylor, 2005; Westcott *et al*., 2009). By gating on the $CD11b^{+}CD115^{+}$ monocytes, I demonstrate that liposomal clodronate preferentially depletes the $7/4^{hi}$ "inflammatory" monocytes as shown in Figure 5.4, F, G and H.

# Figure 5.4

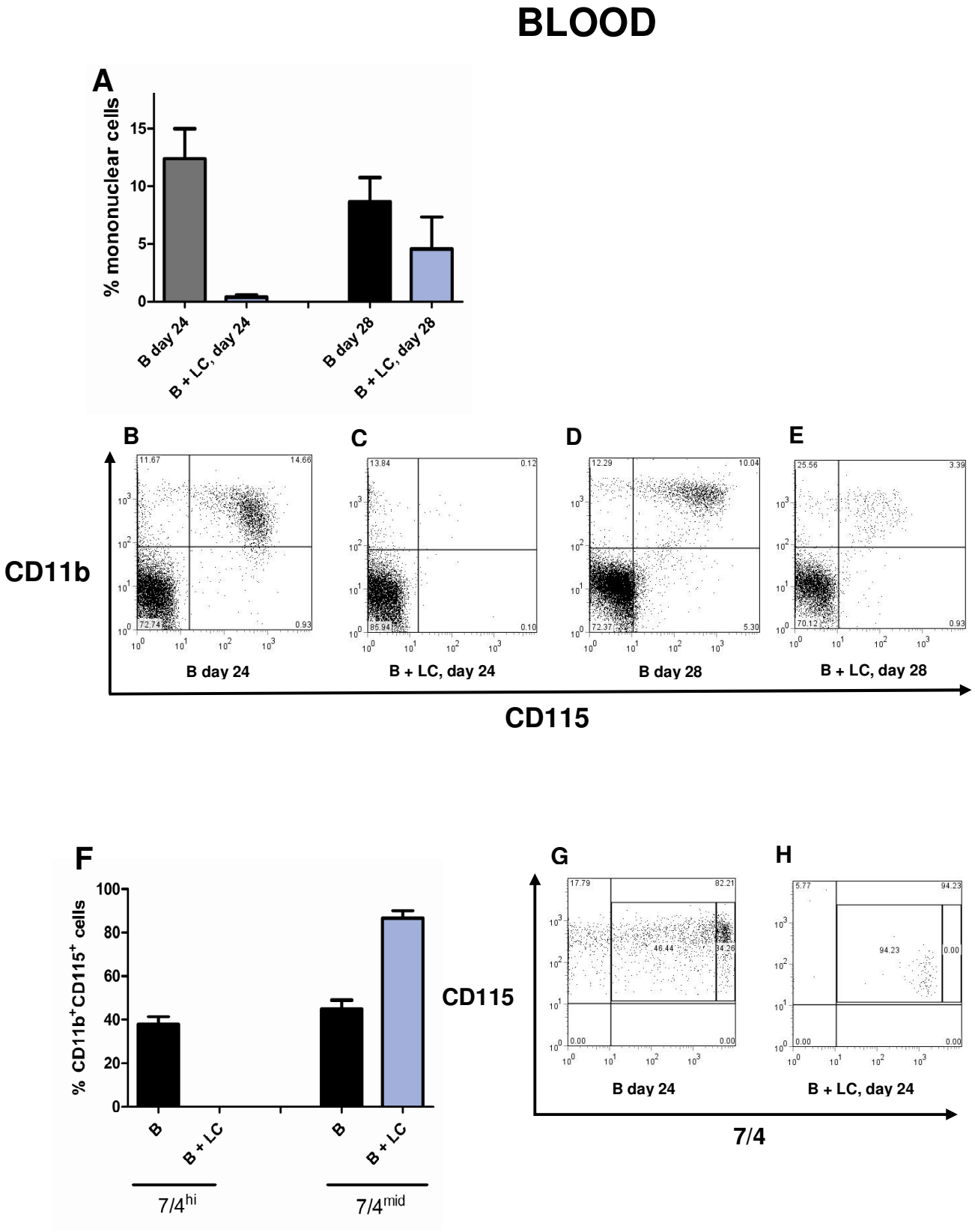

BLOOD
A
% mononuclear cells
15
10
5
0
B day 24
B + LC, day 24
B day 28
B + LC, day 28
B
C
D
E
CD11b
11.67
14.66
72.74
0.93
13.84
0.12
86.94
0.10
12.29
10.04
72.37
5.30
25.56
3.39
70.12
0.93
B day 24
B + LC, day 24
B day 28
B + LC, day 28
CD115
F
% CD11b+CD115+ cells
100
80
60
40
20
0
B
B + LC
B
B + LC
7/4hi
7/4mid
CD115
G
H
17.79
82.21
46.44
34.26
0.00
0.00
5.77
94.23
94.23
0.00
0.00
0.00
B day 24
B + LC, day 24
7/4

Figure 5.4

**Liposomal clodronate preferentially depletes "inflammatory" circulating monocytes**

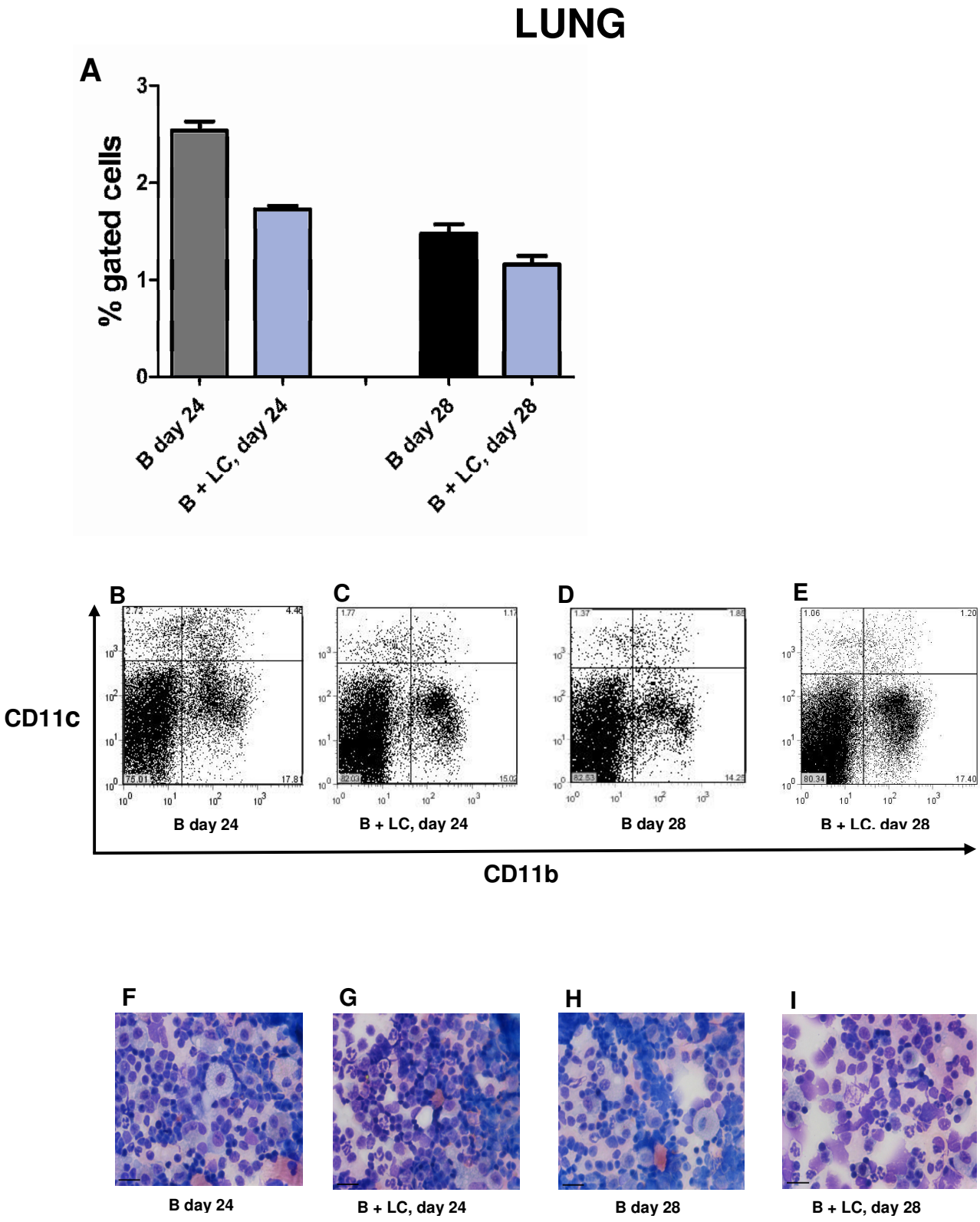
Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl). In addition to bleomycin, groups of mice were also given liposomal clodronate (LC) intra-peritoneally (i.p.) (400µl) on days 21, 22 and 23. Mice were culled on day 24 or day 28. Single cell suspensions were produced from mouse whole blood and analysed by flow cytometry (A)-(E). (A)-(C) Monocytes are significantly depleted 24 hours after the final injection of liposomal clodronate (bleomycin, B, cull day 24 (A), (B) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 24 (A), (C)). (A), (D) and (E) A further 96 hours later monocyte levels are almost back to control levels (bleomycin, B, cull day 28 (A), (D) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 28 (A), (E)). Sub-gating was performed on $CD11b^{+}CD115^{+}$ monocytes (F)-(H). There is complete depletion of $7/4^{hi}$ "inflammatory" monocytes (F)-(H) (bleomycin, B, cull day 24 (F), (G) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 24 (F), (H)) and a relative increase in $7/4^{mid}$ monocytes (F). n=3 per group. Data are presented as mean +/- SEM.

**Systemic depletion of circulating monocytes reduces the proportion of lung macrophages**

Having established that systemic administration of liposomal clodronate depletes circulating monocytes, I wished to establish the effect on the lung macrophage population. To address this question, female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl), liposomal clodronate by intra-peritoneal (i.p.) injection (400µl) on days 21, 22 and 23, and culled on day 24 or day 28. Control groups of mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 24 or day 28. Using flow cytometric analysis of mouse whole lung digests I demonstrate that 24 hours after the final injection of liposomal clodronate there is a reduction in the proportion of lung macrophages as defined by dual CD11c and CD11b positivity, Figure 5.5, A, B and C. This can be seen visually in cytospins created from lung digests, Figure 5.5, F and G. After a further 96 hours the proportion of lung macrophages have almost returned to those of control (Figure 5.5, A, D and E). Again this can be seen visually in cytospins created from lung digests, as shown in Figure 5.5, H and I.

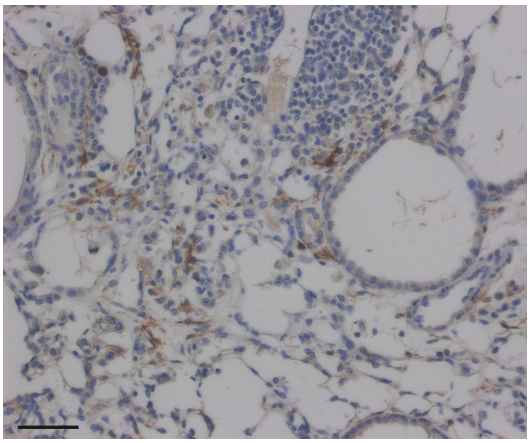
As discussed previously, the F4/80 antigen is a macrophage-restricted marker. As further confirmation of lung macrophage reduction following circulating monocyte depletion, I performed F4/80 immunohistochemistry on lung sections of mice treated as follows: female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl), liposomal clodronate by intra-peritoneal (i.p.) injection (400µl) on days 21, 22 and 23, and culled on day 25. A control group of mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 25. As demonstrated in Figure 5.5, J, lung macrophages stain with F4/80 25 days after bleomycin instillation. When in addition liposomal clodronate is given i.p. (400µl) on days 21, 22 and 23, there is a significant reduction in lung fibrosis and in the degree of F4/80 staining (Figure 5.5K). As shown in Figure 5.5, L-O, there is no staining when an isotype control, or no primary control, is used instead of the F4/80 primary antibody.

# Figure 5.5.1


LUNG
A
% gated cells
3
2
1
0
B day 24
B + LC, day 24
B day 28
B + LC, day 28
B
C
D
E
CD11c
CD11b
B day 24
B + LC, day 24
B day 28
B + LC. dav 28
F
G
H
I
B day 24
B + LC, day 24
B day 28
B + LC, day 28

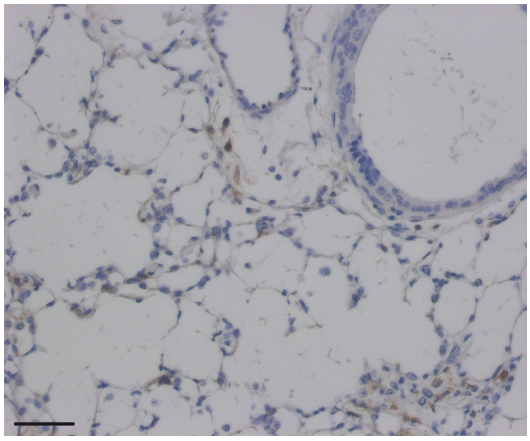
# Figure 5.5.2

J



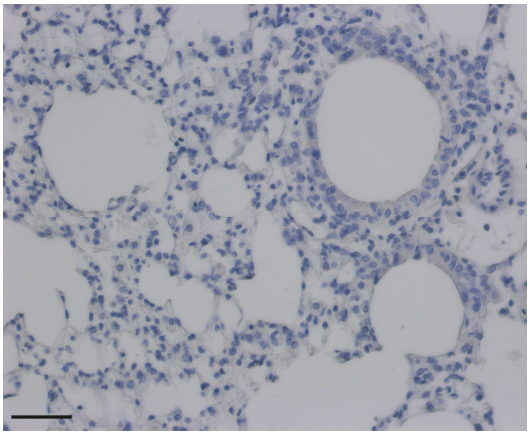
Bleomycin

K



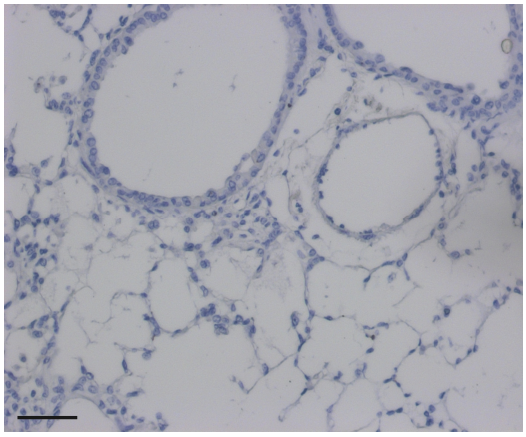
Bleomycin + LC

L



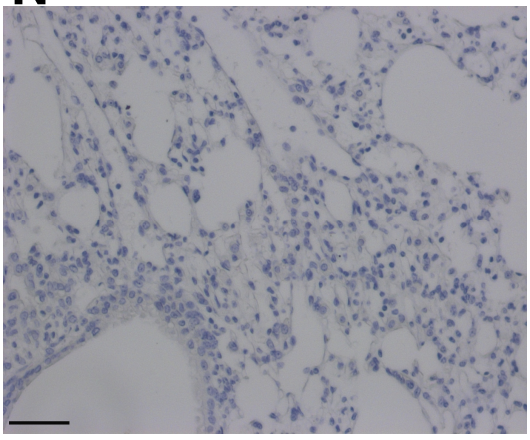
Bleomycin, *Isotype*

M



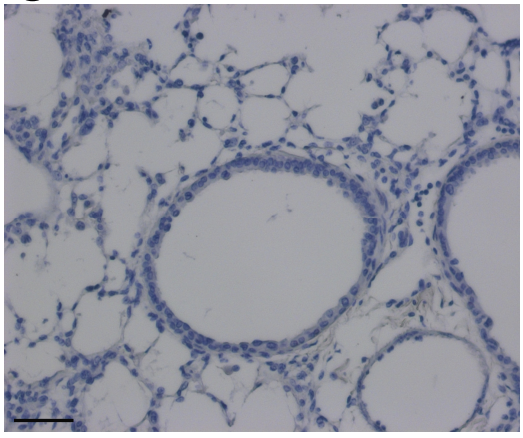
Bleomycin + LC, *Isotype*

N



Bleomycin, *no primary*

O



Bleomycin + LC, *no primary*

Figure 5.5

**Systemic depletion of circulating monocytes reduces the proportion of lung macrophages**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl). In addition to bleomycin, groups of mice were also given liposomal clodronate (LC) intra-peritoneally (i.p.) (400µl) on days 21, 22 and 23. Mice were culled on day 24 (A)-(C), (F), (G), day 25 (J)-(O) or day 28 (A), (D), (E), (H), (I). Single cell suspensions were produced from mouse whole lung digests and analysed by flow cytometry (A)-(E), or cytospins created (F)-(I). Immunohistochemistry was performed lung sections (J)-(O). (A)-(C), (F), (G) There is a reduction in the proportion of lung macrophages 24 hours after the final injection of liposomal clodronate (Bleomycin, B, cull day 24 (A), (B), (F) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 24 (A), (C), (G)). A further 96 hours later the proportion of lung macrophages are almost back to those of control (bleomycin, B, cull day 28 (A), (D), (H) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 28 (A), (E), (I)). (J)-(K) Lung macrophages, as identified by F4/80 staining, are reduced following circulating monocyte depletion. (L)-(M) There is no staining in mice treated with bleomycin +/- liposomal clodronate when an isotype control is used instead of the F4/80 primary antibody. (N)-(O) There is no staining in mice treated with bleomycin +/- liposomal clodronate when a no primary control is used instead of the F4/80 primary antibody. (A)-(I) n=3 per group, (J)-(O) n=5 per group. Data are presented as mean +/- SEM. Bar 200µm.

**Systemic depletion of circulating monocytes reduces the proportion of bronchoalveolar lavage fluid macrophages**

Having established thus far that systemic administration of liposomal clodronate causes depletion of circulating monocytes and leads to a reduction in the proportion of lung macrophages, I wished to establish whether depletion of circulating monocytes had any effect on the proportion of macrophages in the BALF. As a result, female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl), liposomal clodronate by intra-peritoneal (i.p.) injection (400µl) on days 21, 22 and 23, and culled on day 24 or day 28. Control groups of mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 24 or day 28. Using flow cytometric analysis of bronchoalveolar lavage fluid I demonstrate that 24 hours after the final injection of liposomal clodronate there is a reduction in the proportion of BALF macrophages as defined by dual CD11c and CD11b positivity, Figure 5.6, A, B and C. This can be seen visually in cytospins created from BALF, Figure 5.6, F and G, where there is a reduction in the proportion of "foamy" macrophages and an increase in mononuclear cells (macrophages and lymphocytes) following liposomal clodronate administration. After a further 96 hours the proportion of BALF macrophages have almost returned to those of control (Figure 5.6, A, D and E). Again this can be seen visually in cytospins created from BALF as shown in Figure 5.6, H and I.

# Figure 5.6

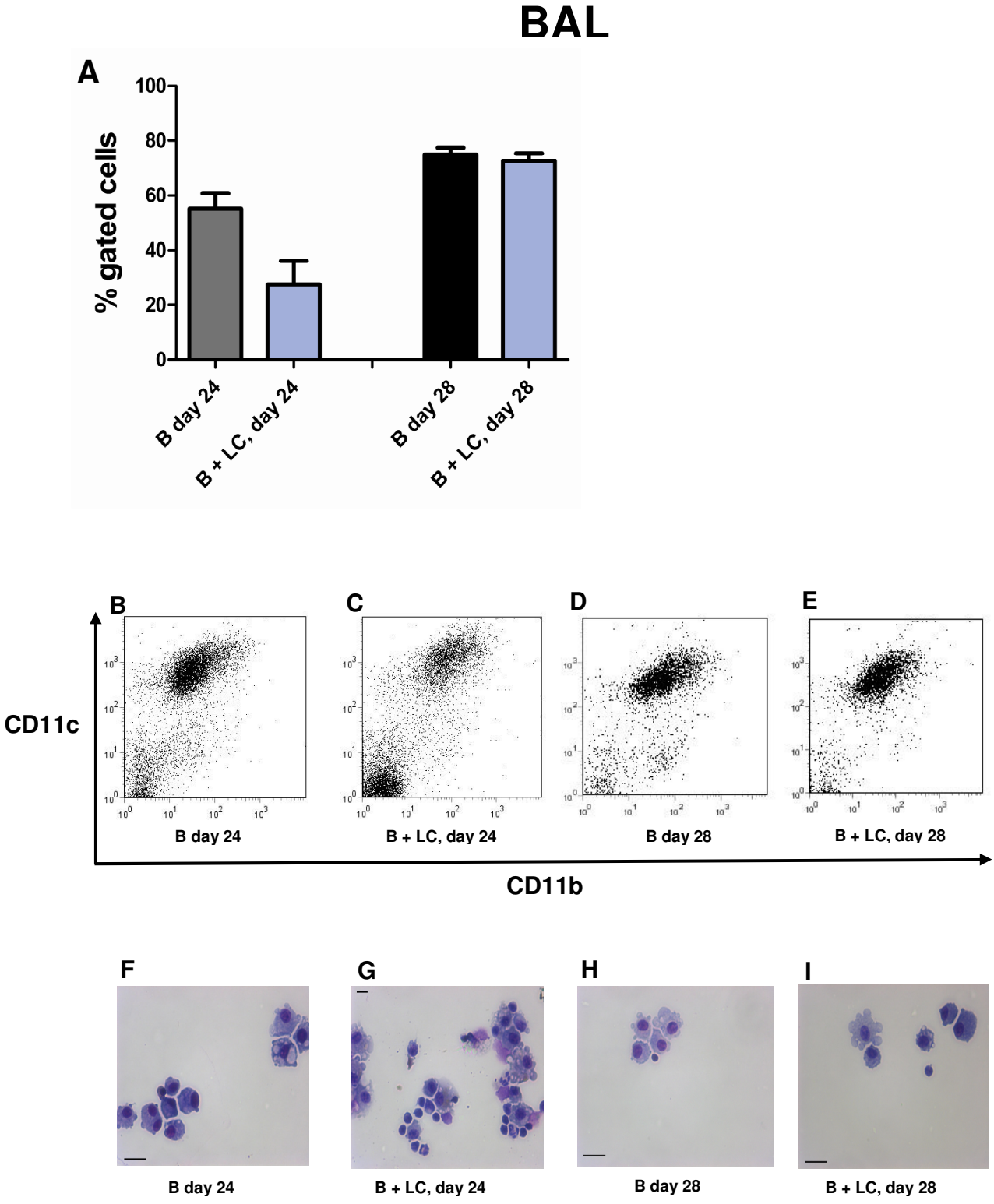

BAL
A
% gated cells
100
80
60
40
20
0
B day 24
B + LC, day 24
B day 28
B + LC, day 28
B
C
D
E
CD11c
CD11b
B day 24
B + LC, day 24
B day 28
B + LC, day 28
F
G
H
I
B day 24
B + LC, day 24
B day 28
B + LC, day 28

Figure 5.6

**Systemic depletion of circulating monocytes reduces the proportion of bronchoalveolar lavage fluid macrophages**

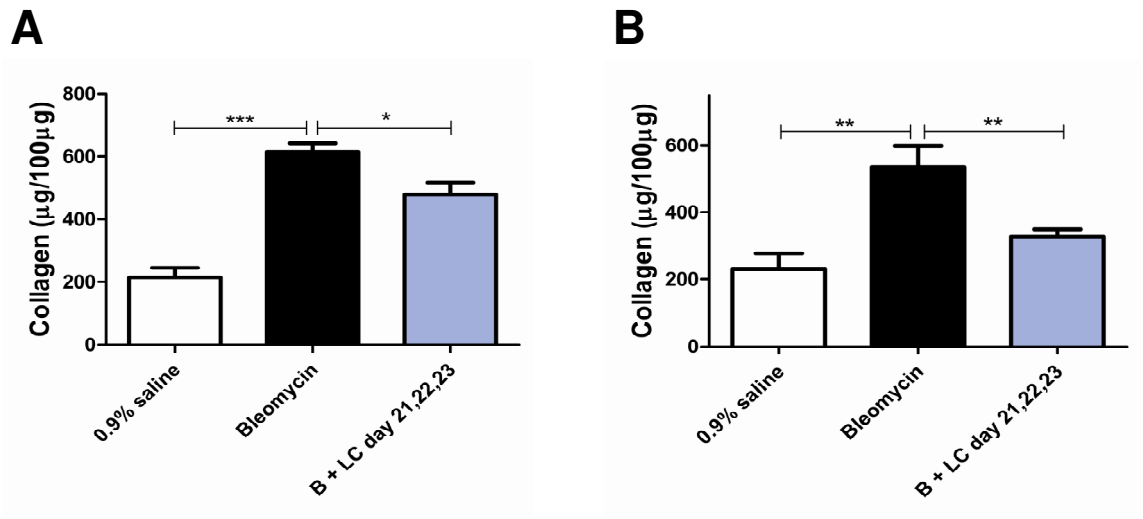
Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl). In addition to bleomycin, groups of mice were also given liposomal clodronate (LC) intra-peritoneally (i.p.) (400µl) on days 21, 22 and 23. Mice were culled on day 24 (A)-(C), (F), (G), or day 28 (A), (D), (E), (H), (I). Single cell suspensions were produced from mouse bronchoalveolar lavage fluid (BALF) and analysed by flow cytometry (A)-(E), or cytospins created (F)-(I). (A)-(C), (F), (G) There is a reduction in the proportion of BALF macrophages 24 hours after the final injection of liposomal clodronate (Bleomycin, B, cull day 24 (A), (B), (F) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 24 (A), (C), (G)). A further 96 hours later the proportion of BALF macrophages are almost back to those of control (bleomycin, B, cull day 28 (A), (D), (H) vs. bleomycin, B, *plus* LC on days 21, 22, 23, cull on day 28 (A), (E), (I)). n=3 per group. Data are presented as mean +/- SEM.

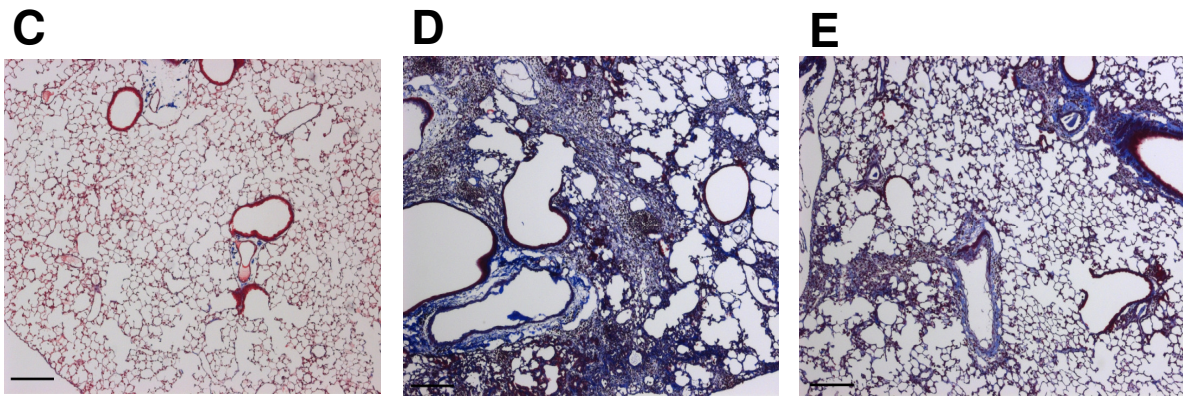
**Circulating monocytes regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model**

Next I wished to evaluate the role of circulating monocytes during the progressive phase of pulmonary fibrosis (day 18 onwards). In our bleomycin model, fibrosis peaks around 28-32 days (Chapter 3, Figure 3.2, A and G, and Figure 3.5, A, B and G). Consequently, female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0 and culled on day 28 or day 32. Circulating monocytes were depleted by giving liposomal clodronate (400µl) by i.p. injection on days 21, 22 and 23. As shown in Figure 5.7, A and B, depletion of circulating monocytes significantly reduces the degree of pulmonary fibrosis that develops as measured by Sircol assay, when mice are culled on either day 28 (Figure 5.7A) or day 32 (Figure 5.7B). This reduction in fibrosis is confirmed when lung sections are stained by Masson's trichrome, as shown in Figure 5.7, C, D and E, where mice were given 0.9% saline and culled on day 28 (Figure 5.7C), bleomycin alone and culled on day 28 (Figure 5.7D) or bleomycin plus liposomal clodronate i.p. on days 21, 22 and 23 and culled on day 28 (Figure 5.7E). Furthermore, qPCR performed on lung homogenates 25 days after bleomycin instillation demonstrated a reduction in expression of the surrogate markers of fibrosis *α-smooth muscle actin* (*α-SMA*) and *Col1A1* after depletion of circulating monocytes (on days 21, 22 and 23) (Figure 5.7, F and G, respectively). Standard curves for the qPCR analyses are shown in Figure 5.7, H-J.

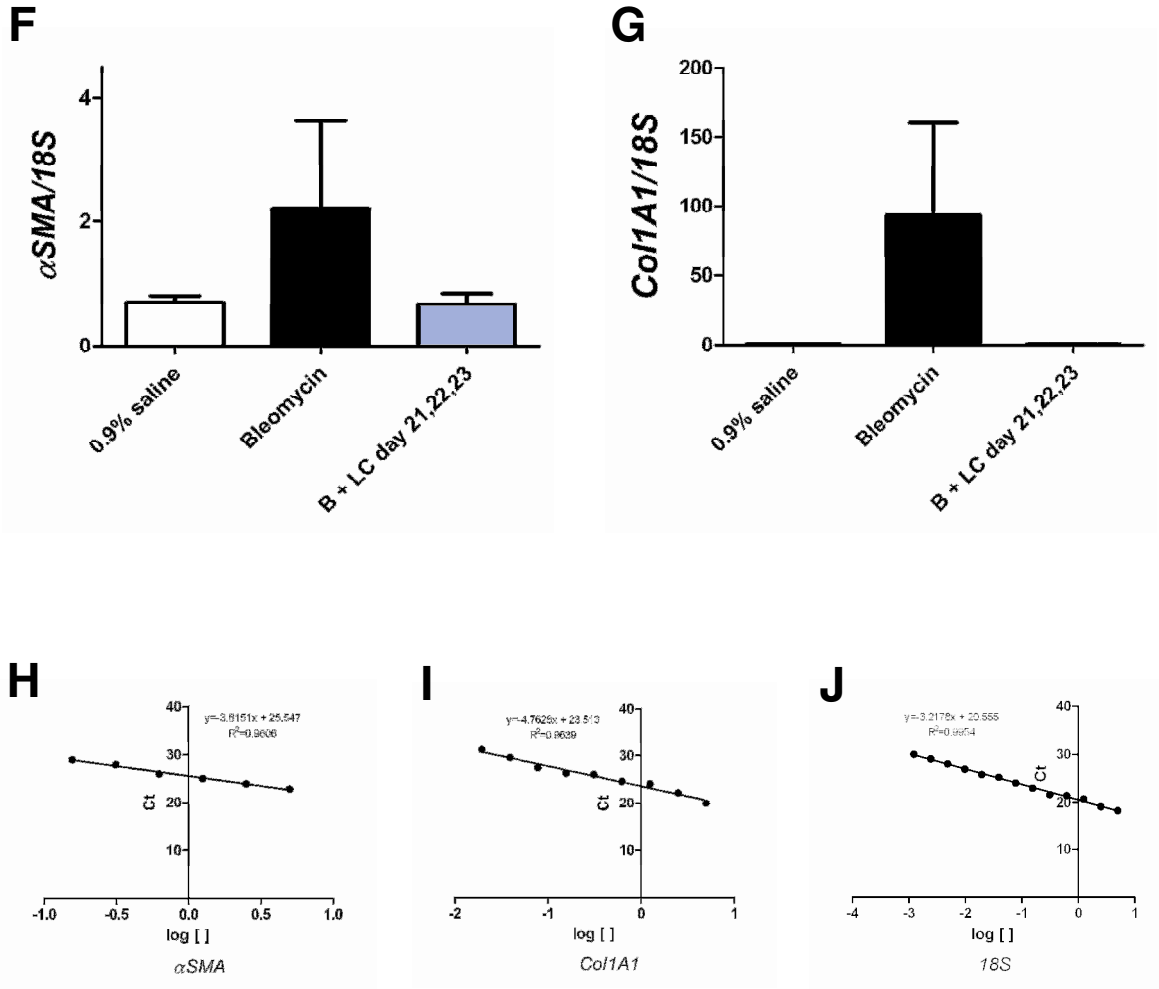
To ensure that this result was mediated by circulating monocyte depletion per se rather than being a liposomal effect, I set up an independent experiment where female C57Bl/6 mice were given 0.033mg bleomycin i.t. alone or 0.033mg bleomycin i.t. on day 0 plus liposomal phosphate-buffered saline (LP) i.p. (400µl) on days 21, 22 and 23 and culled on day 32. As shown in Figure 5.7, K, there is in fact a trend to worsening of pulmonary fibrosis, as measured by Sircol assay, following liposomal PBS injection. Masson's trichrome staining of lung sections (Figure 5.7, L and M) demonstrates that there is certainly no reduction in fibrosis following injection of liposomal PBS.

# Figure 5.7.1


A
B
Collagen (µg/100µg)
800
600
400
200
0
***
*
**
**
0.9% saline
Bleomycin
B + LC day 21,22,23

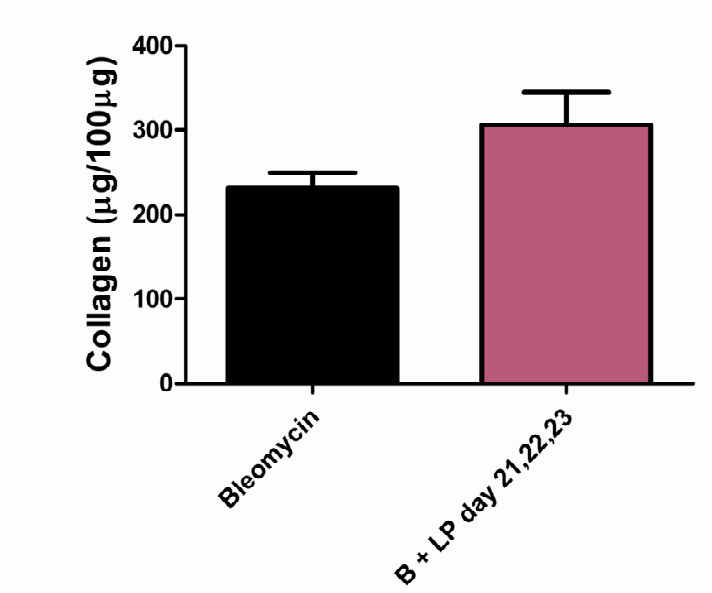

C
D
E
0.9% saline
Bleomycin
B + LC day 21,22,23

# Figure 5.7.2

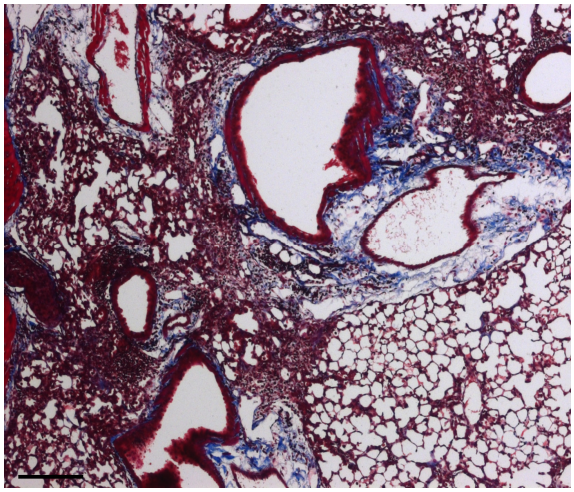

F
αSMA/18S
4
2
0
0.9% saline
Bleomycin
B + LC day 21,22,23
G
Col1A1/18S
200
150
100
50
0
0.9% saline
Bleomycin
B + LC day 21,22,23
H
y=-3.8151x + 25.547
R²=0.9606
Ct
40
30
20
10
-1.0
-0.5
0.0
0.5
1.0
log [ ]
αSMA
I
y=-4.7625x + 23.513
R²=0.9629
Ct
40
30
20
10
-2
-1
0
1
log [ ]
Col1A1
J
y=-3.2178x + 20.555
R²=0.9974
Ct
40
30
20
10
-4
-3
-2
-1
0
1
log [ ]
18S

# Figure 5.7.3

K

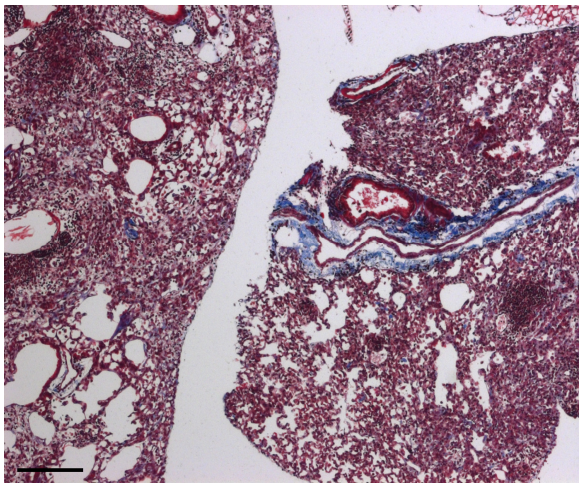



L



Bleomycin

M



B + LP day 21,22,23

Figure 5.7

**Circulating monocytes regulate the progressive fibrotic phase of pulmonary fibrosis in the bleomycin model**

Depletion of circulating monocytes ameliorates lung fibrosis. Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) +/- liposomal clodronate (400µl) by intra-peritoneal (i.p.) injection on days 21, 22 and 23 and culled on day 28 (A), (D), (E), day 32 (B), or day 25 (F), (G). 0.9% saline was given i.t. (50µl) as a control and mice culled on day 28 (C). Bleomycin 0.033mg (50µl) +/- liposomal PBS (LP) i.p. (400µl) on days 21, 22 and 23 were administered to female C57Bl/6 mice in an independent experiment (K)-(M) as a further control, and mice culled on day 32. (A), (B) Mice were given 0.9% saline, bleomycin alone, or bleomycin, B, *plus* liposomal clodronate i.p. (400µl) during progressive fibrosis and culled at peak lung fibrosis (day 28, (A) or day 32 (B)). Depletion of circulating monocytes reduces lung fibrosis as measured by Sircol assay (bleomycin, B, alone vs. bleomycin *plus* liposomal clodronate (LC) on days 21, 22, 23, cull day 28 (A), *p=0.0185; bleomycin, B, vs. bleomycin, B, *plus* LC on days 21, 22 and 23, cull day 32 (B), *p=0.0052). (C)-(E) Masson's trichrome staining on representative lung sections of mice treated with 0.9% saline (C), bleomycin alone (D) or bleomycin *plus* LC on days 21, 22 and 23 (E). (F) and (G) Female C57Bl/6 mice were given bleomycin 0.033mg (50µl) i.t. +/- LC i.p. (400µl) on days 21, 22 and 23 and culled on day 25. qPCR was performed on lung homogenates. (F) Depletion of circulating monocytes leads to a reduction in *α-SMA* gene expression, p=ns. (G) Depletion of circulating monocytes leads to a reduction in *Col1A1* gene expression, p=ns. Representative standard curves used in qPCR analysis are shown for (H) *αSMA*, bleomycin treated lung, (I) *Col1A1*, bleomycin treated lung, and (J) *18S*, bleomycin treated lung. (K)-(M) Female C57Bl/6 mice were given 0.033mg bleomycin i.t.(50µl) alone, or bleomycin, B, *plus* liposomal PBS (LP) i.p. (400µl) on days 21, 22 and 23 and culled on day 32. (K) Administration of bleomycin *plus* liposomal PBS does not reduce fibrosis as measured by Sircol assay, but leads to a trend in worsening of pulmonary fibrosis. (L)-(M) Masson's trichrome staining on representative lung sections of mice

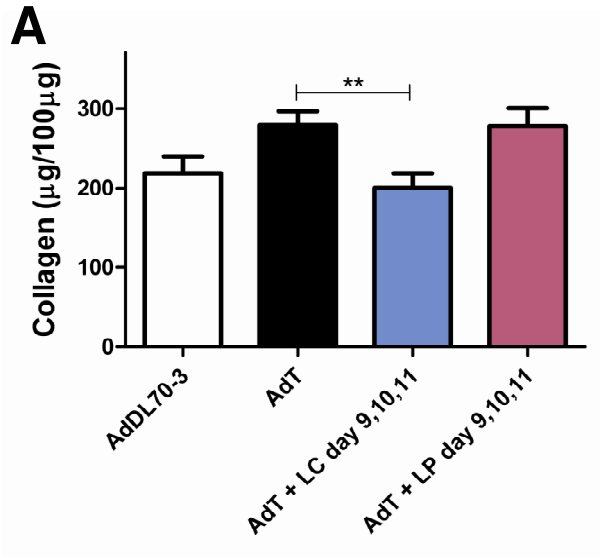
treated with bleomycin alone (L) or bleomycin, B, *plus* liposomal PBS (LP) (days 21, 22 and 23) (M). (A), (C), (D), (E) n=4-5, (B) n=6-8. (F)-(G) n=4-5. (K)-(M) n=5 Data are presented as (A), (B), (F), (G), (K) mean +/- SEM. Bar 200µm.

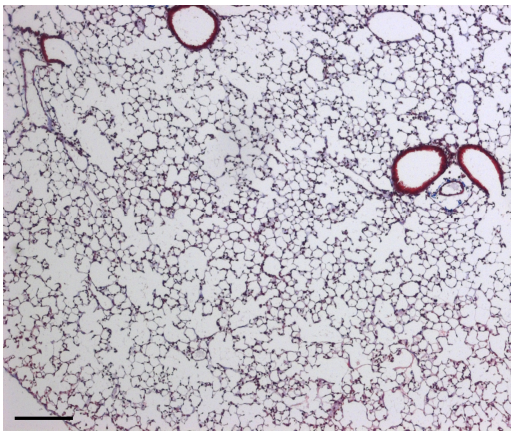
**Circulating monocytes regulate the progressive fibrotic phase of pulmonary fibrosis in the AdTGFβ model**

Having established that circulating monocytes regulate the progressive fibrotic phase of bleomycin-induced pulmonary fibrosis, I wished to back up our findings using a second model. As a result I next sought to determine the effect of depletion of circulating monocytes during the progressive phase of fibrosis in the AdTGFβ model. As discussed earlier, previous work using this model in mice has established that peak fibrosis occurs at around 14 days (Warshamana *et al*., 2002). Our dose finding study (Chapter 3, Figure 3.6B) identified $1x10^8$ pfu of AdTGFβ (50μl) as the optimal dose that could be given to induce significant lung fibrosis without causing undue harm. As a result female C57Bl/6 mice were given $1x10^8$ pfu (50μl) of AdTGFβ (AdT) i.t. on day 0 and culled on day 14. In addition mice were given liposomal clodronate (LC) (400μl) i.p. or liposomal phosphate-buffered saline (LP) (400μl) i.p. on days 9, 10 and 11. Empty adenoviral vector (AdDL70-3, $1x10^8$ pfu (50μl)) was given i.t. as a control and did not induce fibrosis (Figure 5.8, A and B). Depletion of circulating monocytes leads to a reduction in pulmonary fibrosis as measured by Sircol assay (Figure 5.8A). This was confirmed by Masson's trichrome staining of lung sections as can be seen in representative images shown in Figure 5.8, C and D. Administration of liposomal control (liposomal PBS, LP) instead of liposomal clodronate did not reduce pulmonary fibrosis as measured by Sircol assay (Figure 5.8A). In fact there was a suggestion of a worsening of pulmonary fibrosis when lung sections stained by Masson's trichrome were visualised (Figure 5.8E). Furthermore, qPCR performed on lung homogenates demonstrated a significant reduction in expression of the surrogate marker of fibrosis *Col1A1* (Figure 5.8F). There was a non-significant reduction in another surrogate marker of fibrosis, *α-smooth muscle actin* (*α-SMA*) (Figure 5.8G), after depletion of circulating monocytes (on days 9, 10 and 11). Representative standard curves for the qPCR analyses are shown in Figure 5.8, H and I.

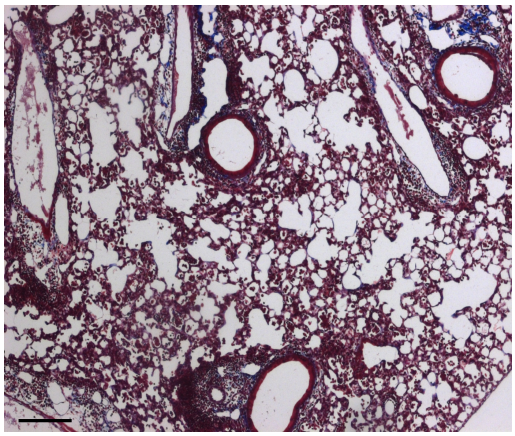
These results back up our findings in the bleomycin model and add further evidence that circulating monocytes are important in promoting the progressive phase of pulmonary fibrosis.

# Figure 5.8.1

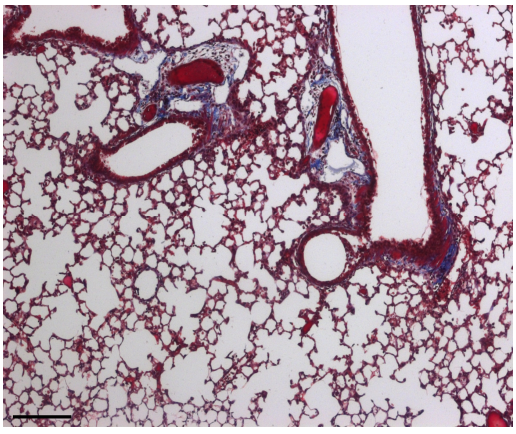


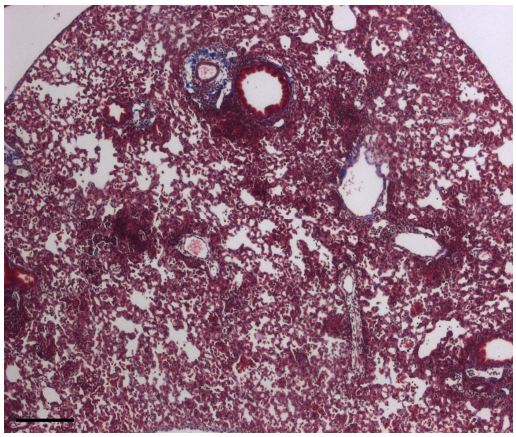

AdDL70-3




AdT




AdT + LC day 9,10,11




AdT + LP day 9,10,11

# Figure 5.8.2

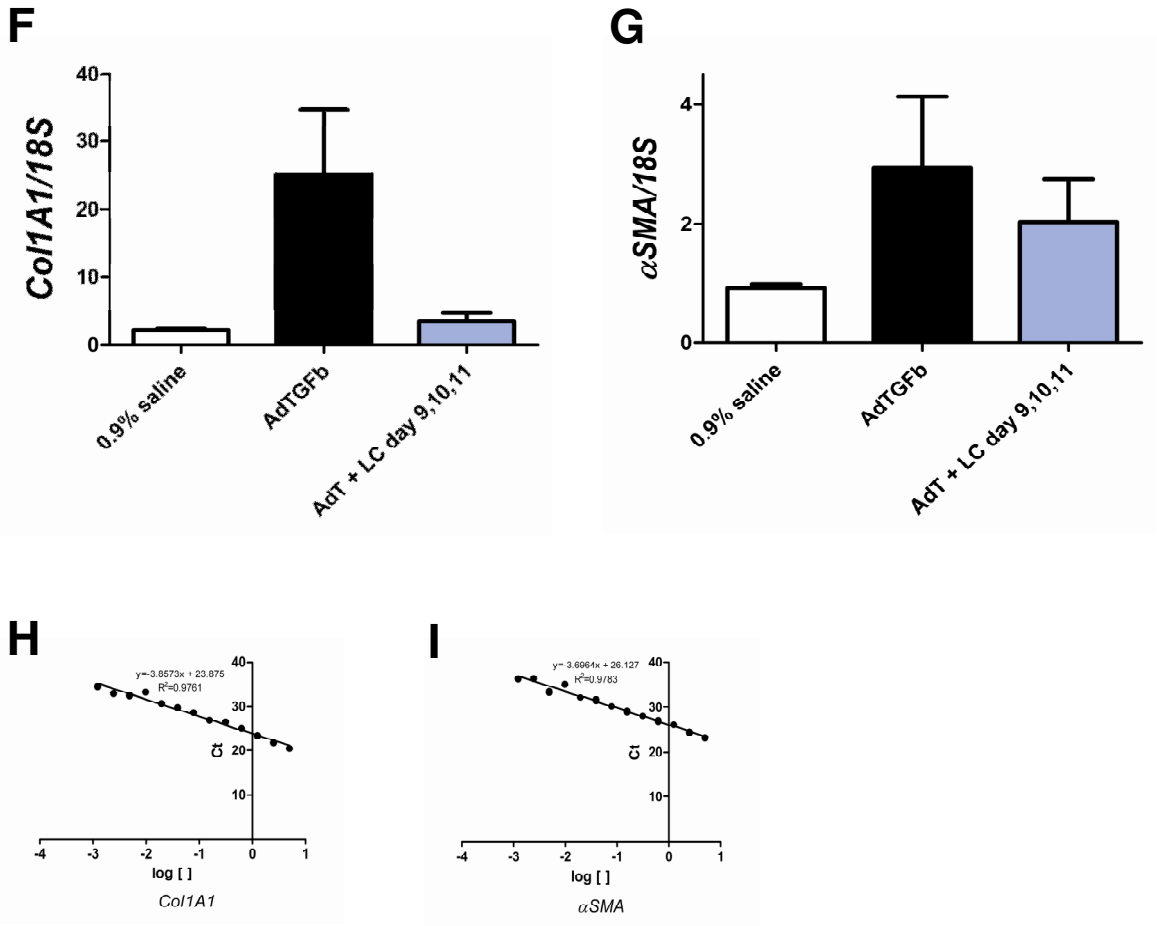

F
Col1A1/18S
40
30
20
10
0
0.9% saline
AdTGFb
AdT + LC day 9,10,11
G
αSMA/18S
4
2
0
0.9% saline
AdTGFb
AdT + LC day 9,10,11
H
y=-3.8573x + 23.875
R²=0.9761
Ct
log [ ]
Col1A1
I
y= 3.6964x + 26.127
R²=0.9783
Ct
log [ ]
αSMA

Figure 5.8

**Circulating monocytes regulate the progressive phase of pulmonary fibrosis in the AdTGFβ model**

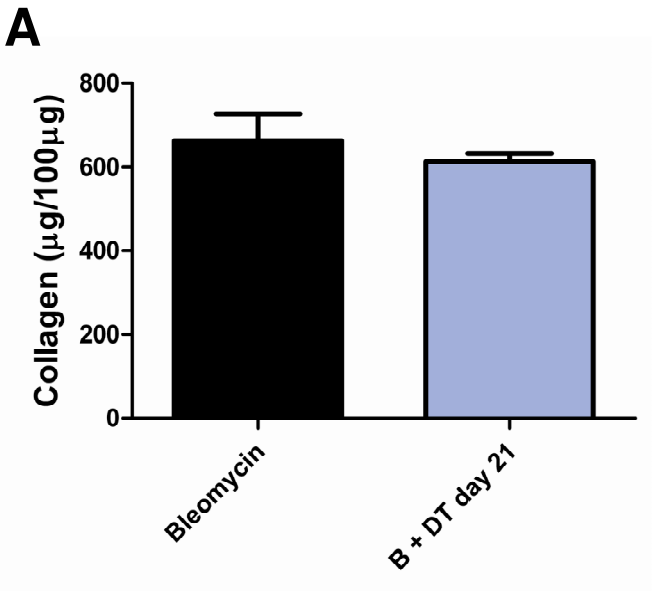
Depletion of circulating monocytes ameliorates lung fibrosis in the AdTGFβ model. Female C57Bl/6 mice were given $1x10^8$ pfu of AdTGFβ i.t. (50μl) and fibrosis quantified by Sircol assay (A). AdDL70-3 ($1x10^8$ pfu/50μl) was given as a control. Masson's trichrome staining was performed on lung sections (B)-(E). qPCR was performed on lung homogenates (F)-(G). (A) Mice were given AdDL70-3, AdTGFβ alone, AdTGFβ (AdT) plus liposomal clodronate (LC) i.p. (400μl) on days 9, 10 and 11, or AdTGFβ (AdT) plus liposomal PBS (LP) i.p. (400μl) on days 9, 10 and 11, and culled at peak lung fibrosis (day 14). Depletion of circulating monocytes reduces lung fibrosis as measured by Sircol assay (AdTGFβ alone vs. AdT *plus* LC on days 9, 10 and 11, **p=0.0091). Administration of liposomal PBS as a control instead of liposomal clodronate does not reduce lung fibrosis. (B) AdDL70-3 does not induce significant fibrosis at 14 days. (C) AdTGFβ induces significant fibrosis by 14 days. (D) Depletion of circulating monocytes ameliorates lung fibrosis. (E) Administration of liposomal PBS does not reduce fibrosis. (F) Depletion of circulating monocytes leads to a reduction in *Col1A1* gene expression, p=ns. (G) Depletion of circulating monocytes leads to a trend in reduction in *α-SMA* gene expression, p=ns. Representative standard curves used in qPCR analysis are shown for (H) *Col1A1*, AdTGFβ treated lung, and (I) *αSMA*, AdTGFβ treated lung. (A)-(E) n=5-7, (F)-(G) n=5-6. Data are presented as mean +/- SEM. Bar 200μm.

**Circulating monocyte depletion using the CD11b-DTR system does not regulate the progressive phase of pulmonary fibrosis in the bleomycin model**

So far in this chapter I have used liposomal clodronate to deplete circulating monocytes. I wished to establish whether use of another depletional strategy would have the same effect. The CD11b-DTR transgenic mouse was generated as a means of studying macrophage function *in vivo* (Cailhier *et al*., 2005; Duffield *et al*., 2005). It relies on the fact that the mouse diphtheria toxin (DT) receptor (DTR) binds DT poorly (Naglich *et al.*, 1992). A construct was generated in which the CD11b promoter was upstream of the DTR-eGFP fusion gene (Duffield *et al*., 2005). Downstream of these elements was part of the human growth hormone gene, inserted specifically to provide the necessary splicing and poly-adenylation sequences. CD11b is expressed on both macrophages and granulocytes, but DT administration to CD11b-DTR mice does not deplete granulocytes (Cailhier *et al*., 2005), possibly as a result of their lower levels of protein synthesis (Cailhier *et al*., 2005). The CD11b-DTR transgenic system therefore allows for conditional and specific depletion of monocytes and macrophages. The CD11b-DTR mice were kindly supplied by Dr Jeremy Hughes, MRC/University of Edinburgh Centre for Inflammation Research, Edinburgh.

Female C57Bl/6 CD11b-DTR mice were given 0.033mg bleomycin i.t. (50μl). In addition to this one group of mice was also given DT 10ng/g body weight i.p. on day 21. Mice were culled on day 28. As demonstrated in Figure 5.9, A, B and C, depletion of circulating monocytes does not affect pulmonary fibrosis as quantified by Sircol assay (Figure 5.9A), or as visualised by Masson's trichrome staining of lung sections (Figure 5.9, B and C).

# Figure 5.9


A
800
600
400
200
0
Collagen (µg/100µg)
Bleomycin
B + DT day 21

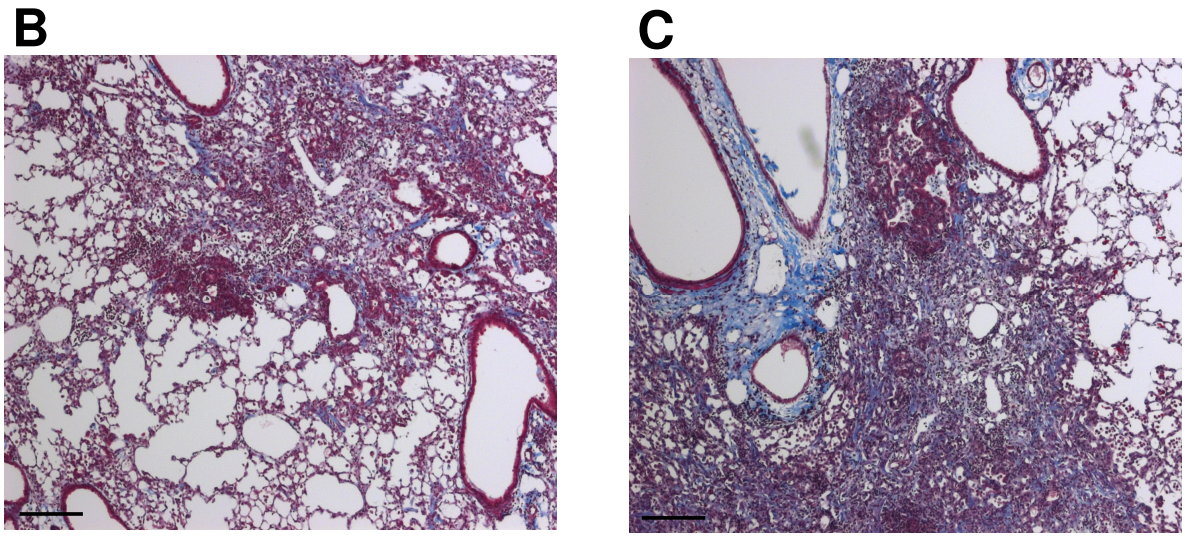

B
C
Bleomycin
B + DT day 21

Figure 5.9

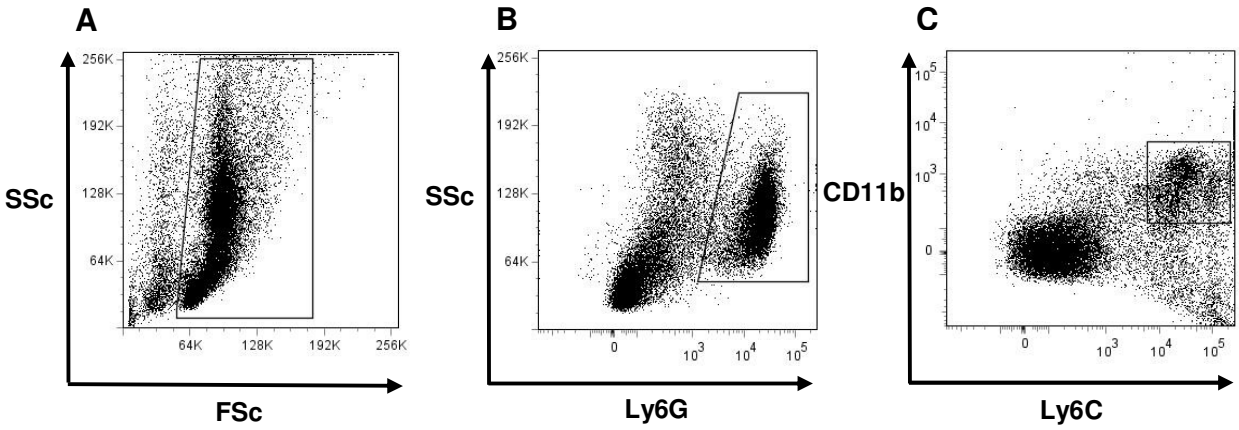
**Circulating monocyte depletion using the CD11b-DTR system does not regulate the progressive phase of pulmonary fibrosis in the bleomycin model**

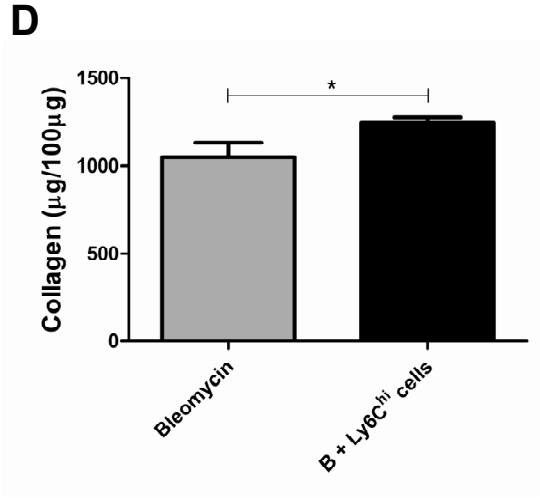
Female C57Bl/6 CD11b-DTR mice were given 0.033mg bleomycin i.t. (50µl) +/- DT i.p. (10ng/g body weight) on day 21. All mice were culled on day 28. Fibrosis was quantified by Sircol assay (A). Masson's trichrome staining was performed on lung sections (B)-(C). (A) Depletion of circulating monocytes by DT has no effect on lung fibrosis as measured by Sircol assay (bleomycin alone vs. bleomycin *plus* DT on day 21, p=ns). (B) and (C) Depletion of circulating monocytes by DT does no affect the degree of pulmonary fibrosis (bleomycin alone (B) vs. bleomycin *plus* DT on day 21 (C)). (A)-(C) n=5-6. Data are presented as mean +/- SEM. Bar 200µm.

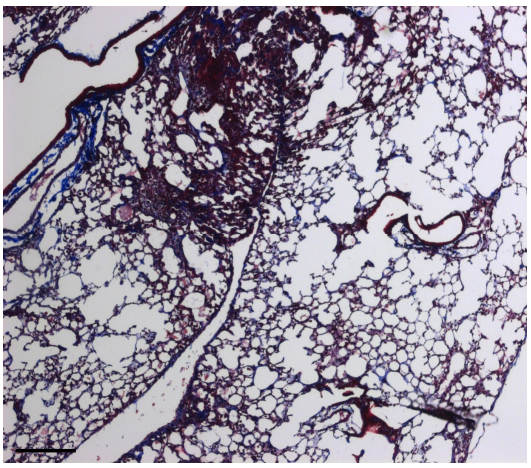
**Adoptive transfer of Ly6C$^{hi}$ monocytes exacerbates pulmonary fibrosis in the bleomycin model**

Our earlier experiments have shown that liposomal clodronate administration i.p. preferentially depletes circulating "inflammatory" monocytes (Figure 5.4). I wished to establish whether adoptive transfer of bone marrow Ly6C$^{hi}$ "inflammatory" monocytes would have any effect on the degree of fibrosis in mice already treated with low-dose bleomycin. Ly6C is expressed on both monocytes and granulocytes, whereas only granulocytes also express Ly6G (Tacke & Randolph 2006). Female C57Bl/6 mice were given 0.0167mg bleomycin i.t. (50µl), 1x10$^6$ Ly6C$^{hi}$ monocytes by tail-vein injection on day 21 and culled on day 28. Ly6C$^{hi}$ monocytes were purified from bone marrow using a BD FACS Aria II (Special order system). As shown in Figure 5.10, live cells were sorted (A), Ly6G$^+$ cells subsequently positively sorted to eliminate granulocytes (B), then Ly6C$^{hi}$ monocytes were sorted from this negatively sorted population (C). As shown in Figure 5.10D, adoptive transfer of these Ly6C$^{hi}$ monocytes exacerbates pulmonary fibrosis as quantified by Sircol assay, and as visualised by Masson's trichrome staining of lung sections (Figure 5.10, E and F). It is interesting to point out that there appears to be profound peri-vascular (as well as interstitial) fibrosis, as shown in Figure 5.10F.

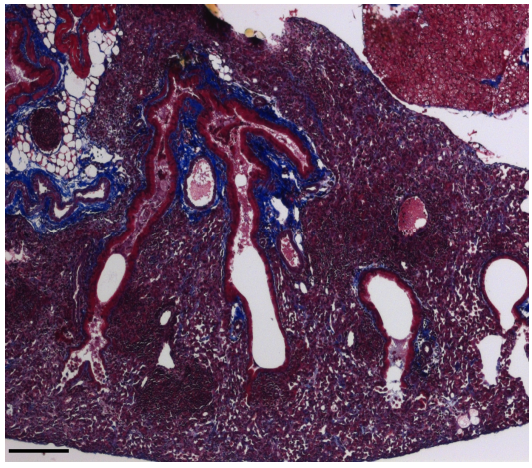
# Figure 5.10




Bleomycin




B + Ly6C$^{hi}$ cells

Figure 5.10

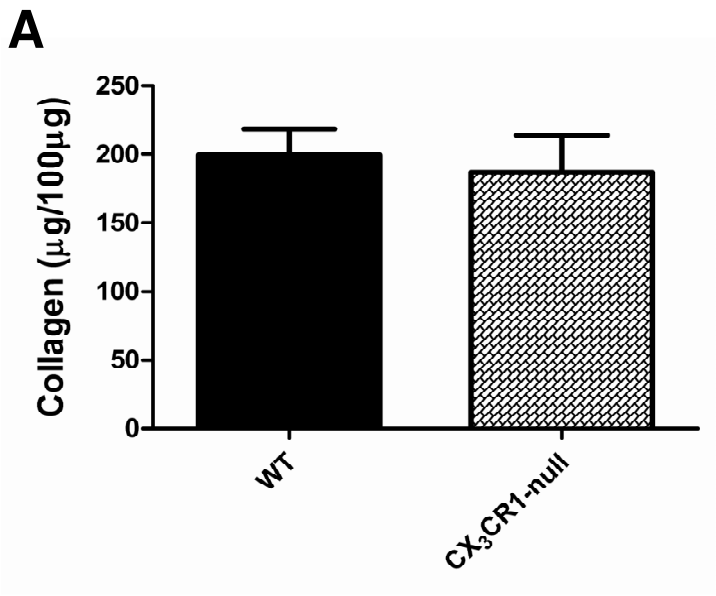
**Adoptive transfer of Ly6C$^{hi}$ monocytes exacerbates pulmonary fibrosis in the bleomycin model**

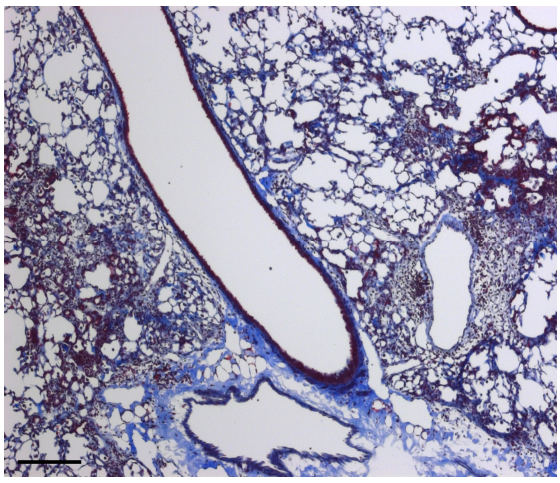
Female C57Bl/6 mice were given 0.0167mg bleomycin i.t. (50µl). Ly6C$^{hi}$ cells were sorted from bone marrow; live cells were first gated (A) and Ly6G$^{+}$ granulocytes positively excluded (B). From the resulting negative fraction Ly6C$^{hi}$ monocytes were positively sorted (C). 1x10$^{6}$ cells were injected into the tail vein of mice on day 21. A control group of mice were injected with media only into the tail vein on day 21. All mice were culled on day 28. Fibrosis was quantified by Sircol assay (D) and visualised by Masson's trichrome staining on lung sections (E), (F). (D) Mice injected with Ly6C$^{hi}$ monocytes have enhanced fibrosis when compared with control (bleomycin *plus* media vs. bleomycin, B, *plus* Ly6C$^{hi}$ monocytes on day 21, *p=0.0304). (E) and (F) Mice injected with Ly6C$^{hi}$ monocytes have more fibrosis, as visualised on Masson's trichrome stained lung sections, than mice injected with media alone. (D)-(F) n=6-8. Data are presented as mean +/- SEM. Bar 200µm.

**$CX_3CR1$-null mice develop pulmonary fibrosis similar to wild-type**

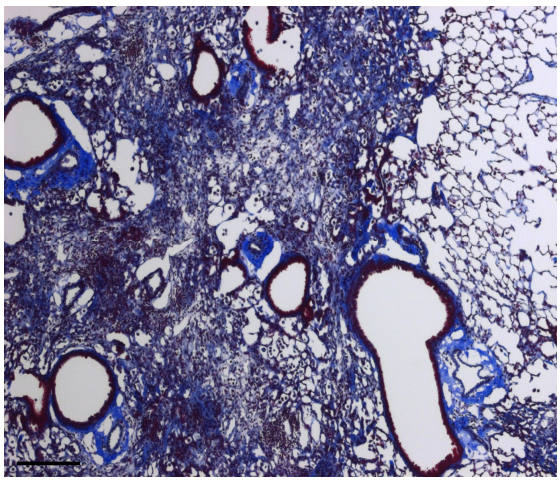
The different monocyte populations in mice have already been described in detail. Briefly, there exist two main populations, the $Ly6C^{lo}$, $CCR2^{-}$, $CX_3CR1^{+}$, $7/4^{mid}$ and $Ly6C^{hi}$, $CCR2^{+}$, $CX_3CR1^{lo}$, $7/4^{hi}$ populations. I have already established that the $Ly6C^{hi}$ population enhances pulmonary fibrosis in the bleomycin model. I wished to establish whether $CX_3CR1$-null mice had an altered lung fibrotic response to bleomycin. These mice were kindly supplied by Professor Christopher Gregory, MRC/University of Edinburgh Centre for Inflammation Research, Edinburgh. Female C57Bl/6 $CX_3CR1$-null mice were given 0.033mg bleomycin i.t. (50µl) and culled on day 32. Wild-type littermates were used as a control. There was no difference in fibrosis between the two groups, as measured by Sircol assay (Figure 5.11A). In addition, there was no obvious difference in fibrosis as visualised by Masson's trichrome staining of lung sections (Figure 5.11, B and C).

# Figure 5.11




WT, Bleomycin




$CX_3CR1$-null, Bleomycin

Figure 5.11

**$CX_3CR1$-null mice develop pulmonary fibrosis similar to wild-type**

Female C57Bl/6 $CX_3CR1$-null mice and their wild-type (WT) littermates were given 0.033mg bleomycin i.t. (50µl) and culled on day 32. Fibrosis was quantified by Sircol assay (A). Masson's trichrome staining was performed on lung sections (B)-(C). (A) $CX_3CR1$-null mice develop pulmonary fibrosis to a similar degree to their wild-type littermates. (B) and (C) No significant difference in fibrosis was observed when lung sections were stained by Masson's trichrome; wild-type mice (B) compared with $CX_3CR1$-null mice (C). (A)-(C) n=6. Data are presented as mean +/- SEM. Bar 200µm.

**Alternatively activated macrophages characterise the progressive fibrotic phase of pulmonary fibrosis in mice**

I have demonstrated in chapter 4 that depletion of lung macrophages results in a reduction in markers of alternative macrophage activation at both the gene and protein level. It has recently been shown that Ly6C$^{hi}$ monocytes have increased expression of alternative macrophage markers in both mice (*Ym1* [*chitinase 3-like protein 3, Chi3l3*]) and humans (*CD163*) (Ingersoll *et al*., 2010). I have shown that CD163 is up-regulated on human alveolar macrophages in patients with IPF, and furthermore it has just been published that in patients with an exacerbation of IPF there is an increase in the number of CD14$^{+}$ monocytes that express CD163 compared to healthy controls (Murray *et al*., 2010).

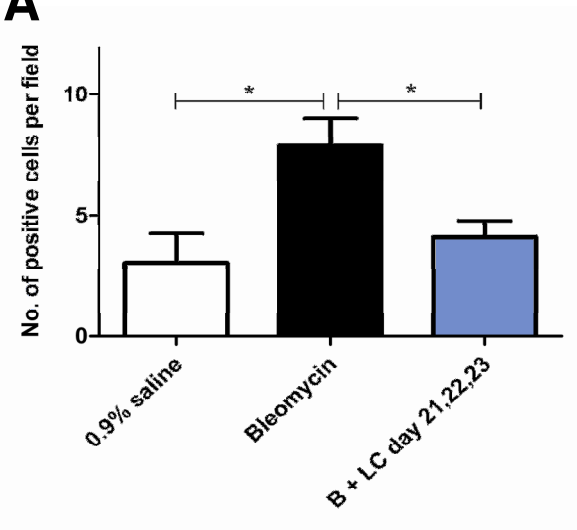
I wished to establish whether there was any correlation between depletion of (Ly6C$^{hi}$) circulating monocytes and alternative macrophage activation state within the lungs. Female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0 with or without liposomal clodronate (400µl) i.p. on days 21, 22 and 23, and culled on day 28. 0.9% saline (50µl) was given i.t. as a control. Quantitative immunohistochemical analysis on lung sections demonstrated a reduction in Ym1 protein expression following circulating monocyte depletion (Figure 5.12, A, B and C). Isotype control and no primary control showed no positive staining in the context of bleomycin-induced lung fibrosis (Figure 5.12, C and D).

Having established an effect at the protein level, I wished to establish whether this effect was also seen at the RNA level. Female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0, with or without liposomal clodronate (400µl) i.p. on days 21, 22 and 23, and culled on day 25. qPCR was performed on lung homogenates for markers of alternative macrophage activation. As shown in Figure 5.12, F and G, depletion of circulating monocytes leads to a reduction in gene expression of characteristic markers of alternative macrophage activation, *Ym1* and *Arginase1*, respectively. Representative standard curves for the qPCR analyses are shown in Figure 5.12, H and I. This data

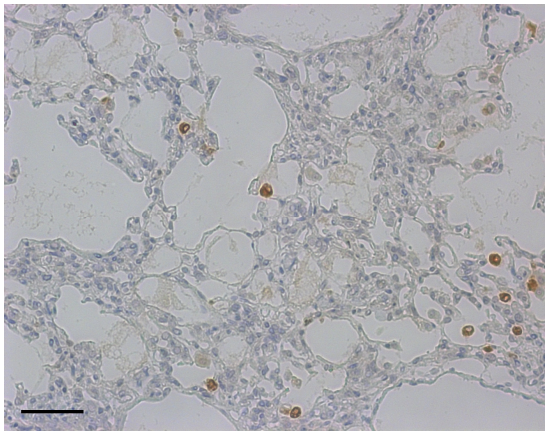
suggests that there is an association between Ly6C$^{hi}$ monocytes and the development of an alternatively activated macrophage environment within the lung during the progressive phase of pulmonary fibrosis.

# Figure 5.12.1

A

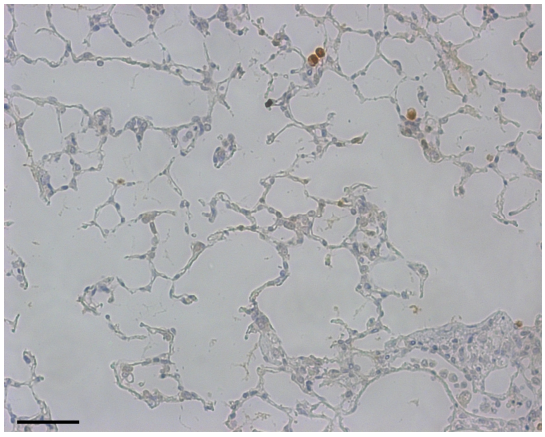



B



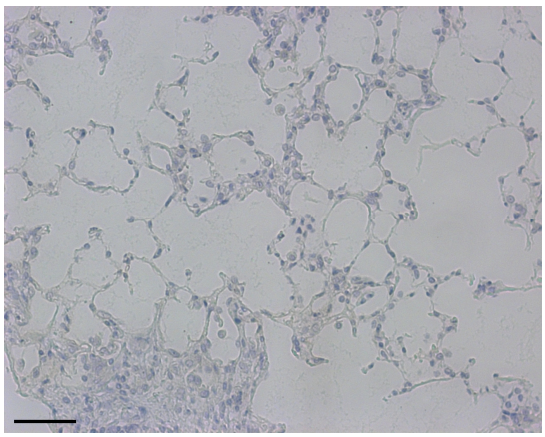
Bleomycin

C



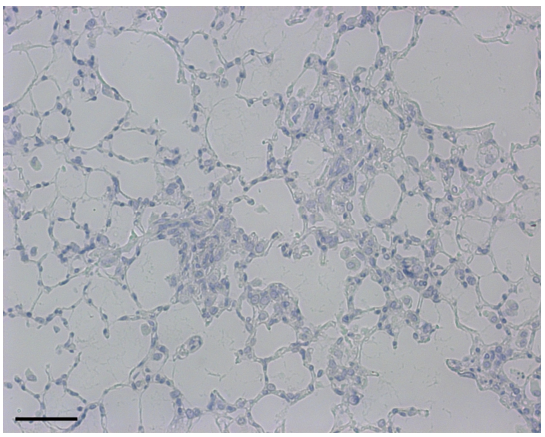
B + LC day 21, 22, 23

D



Bleomycin, *Isotype*

E



Bleomycin, *no primary*

# Figure 5.12.2

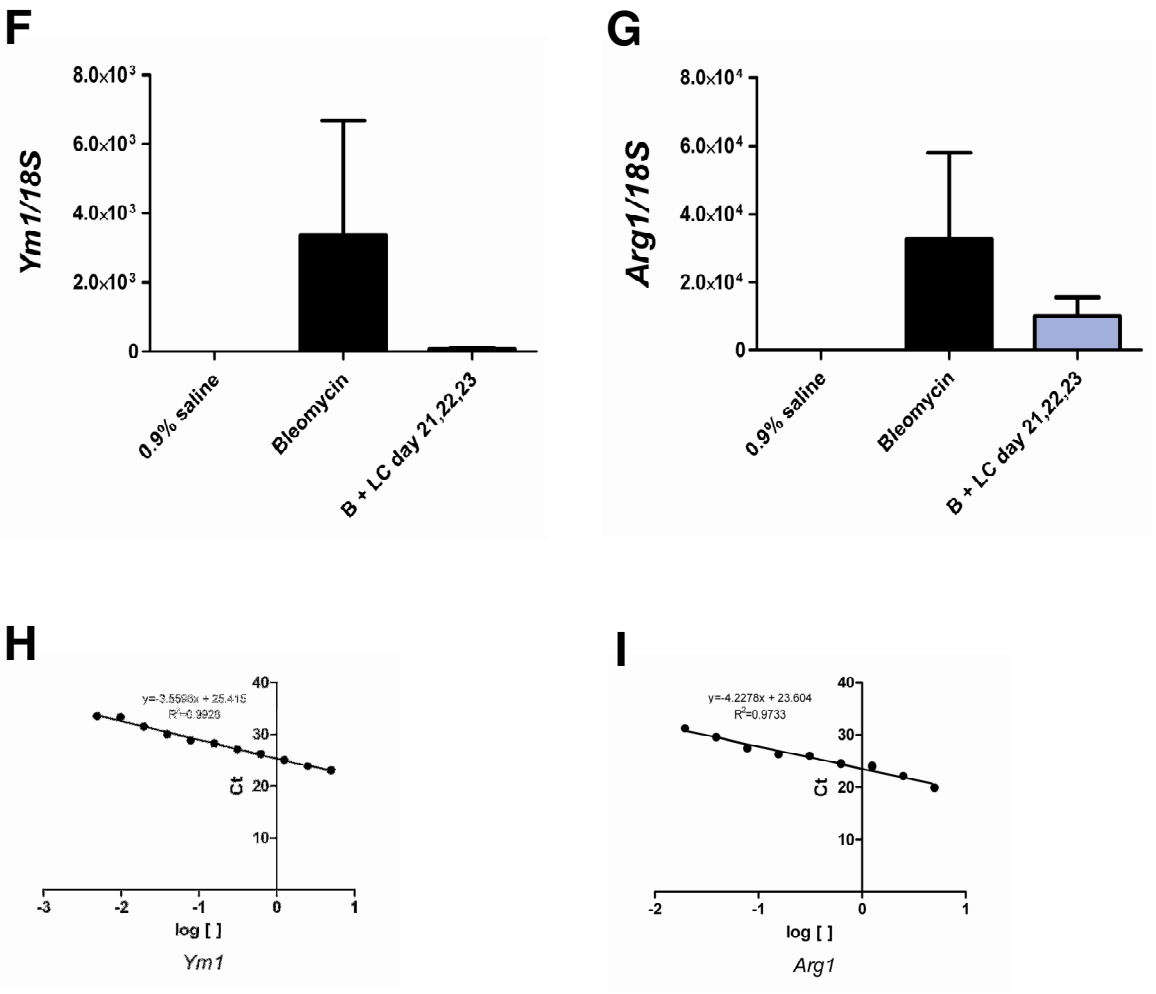

F
8.0x10³
6.0x10³
4.0x10³
2.0x10³
0
Ym1/18S
0.9% saline
Bleomycin
B + LC day 21,22,23
G
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
0
Arg1/18S
0.9% saline
Bleomycin
B + LC day 21,22,23
H
y=-3.5568x + 25.415
R²=0.9928
Ct
log [ ]
Ym1
I
y=-4.2278x + 23.604
R²=0.9733
Ct
log [ ]
Arg1

Figure 5.12

**Alternatively activated macrophages characterise the progressive fibrotic phase of pulmonary fibrosis in mice**

Female C57Bl/6 mice were given bleomycin i.t. (50µl). Liposomal clodronate (LC) was given i.p. (400µl) on days 21, 22 and day 23. Mice were culled on day 28 (immunohistochemistry) or day 25 (qPCR). 0.9% saline was given i.t. as a control. Immunohistochemistry (IHC) was performed on lung sections (A)-(E). qPCR was performed on lung homogenates (F)-(G). (A)-(C) Ym1 expressing lung macrophages are reduced following circulating monocyte depletion (bleomycin alone (A), (B) vs. bleomycin, B, *plus* LC on days 21, 22 and 23, *p=0.0310 (A), (C)). (D) There is no positive staining when an isotype control is used instead of primary antibody. (E) There is no positive staining when a no primary control is used instead of primary antibody. (F) *Ym1* gene expression is reduced following circulating monocyte depletion (bleomycin alone vs. bleomycin, B, *plus* LC on days 21, 22 and 23, p=ns). (G) *Arginase1* (*Arg1*) gene expression is reduced following circulating monocyte depletion (bleomycin alone vs. B *plus* LC on days 21, 22 and 23, p=ns). Representative standard curves used in qPCR analysis are shown for (H) *Ym1* and (I) *Arg1*. (A)-(E) n=4-6, (F)-(G) n=4-5. Data are presented as mean +/- SEM. Bar 200µm.

**Circulating monocytes contribute towards creating a matrix-promoting micro-environment within lung tissue**

As discussed previously the MMP/TIMP ratio is known to be critical for ensuring an appropriate wound healing response and contributes significantly to promotion and resolution of pulmonary fibrosis. I demonstrated in chapter 4 that lung macrophages create a matrix-promoting micro-environment in lung tissue. I wished to establish whether circulating monocytes contributed to the matrix micro-environment within lung tissue, and if so, what effects they have.

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. on day 0 followed by liposomal clodronate (400µl) i.p. on days 21, 22 and 23. Mice were culled on day 25. As shown in Figure 5.13, A and B, depletion of circulating monocytes leads to a reduction in *TIMP1* gene expression and an increase in *MMP2* gene expression, creating a micro-environment favouring matrix degradation. Representative standard curves for the qPCR analyses are shown in Figure 5.13, C and D. This suggests that circulating monocytes contribute towards the creation of a micro-environment that favours matrix-production within lung tissue during the progressive fibrotic phase of bleomycin-induced lung fibrosis.

# Figure 5.13

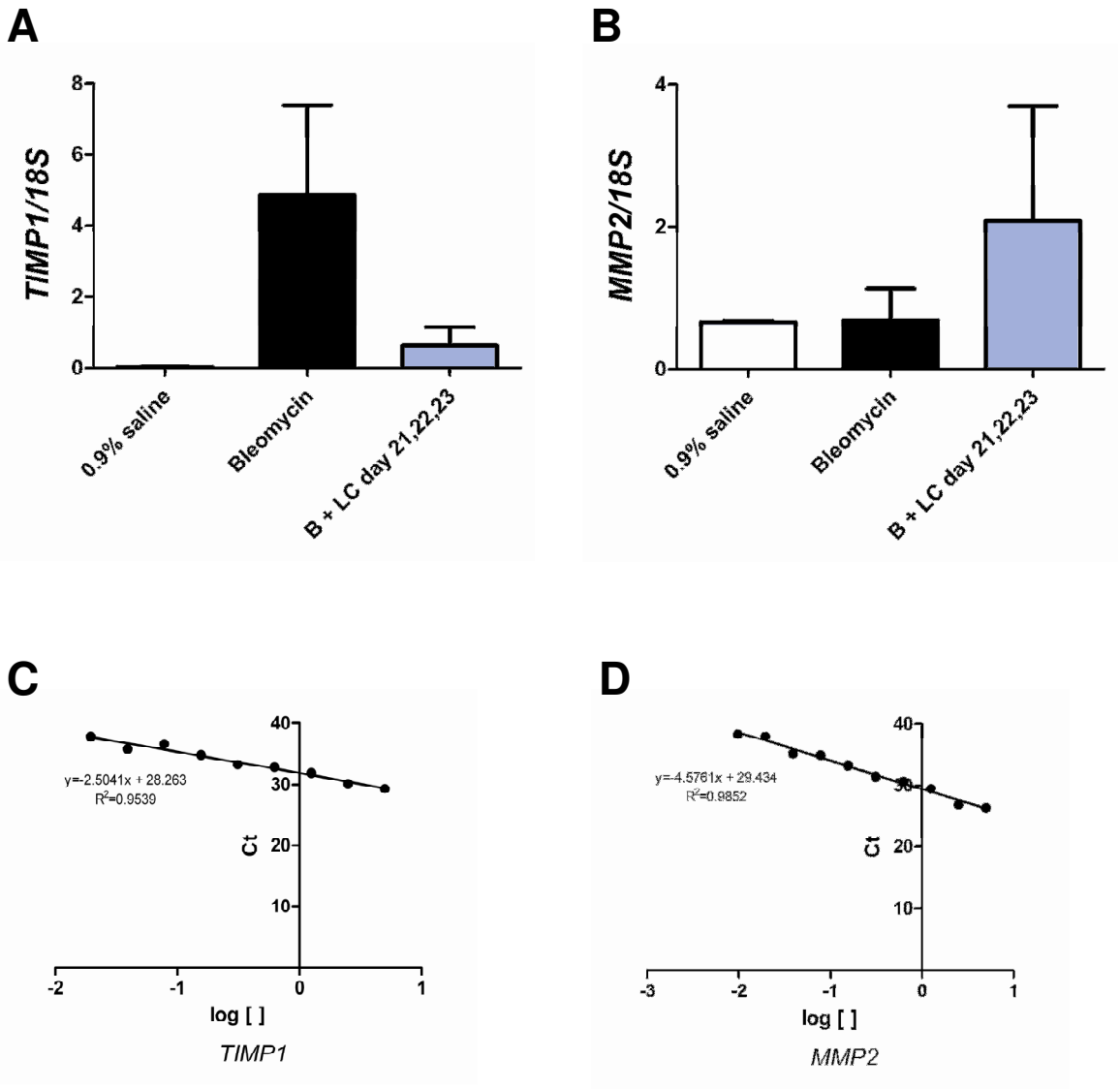

A
TIMP1/18S
8
6
4
2
0
0.9% saline
Bleomycin
B + LC day 21,22,23
B
MMP2/18S
4
2
0
0.9% saline
Bleomycin
B + LC day 21,22,23
C
y=-2.5041x + 28.263
R²=0.9539
Ct
40
30
20
10
-2
-1
0
1
log [ ]
TIMP1
D
y=-4.5761x + 29.434
R²=0.9852
Ct
40
30
20
10
-3
-2
-1
0
1
log [ ]
MMP2

Figure 5.13

**Circulating monocytes contribute towards creating a matrix-promoting microenvironment within lung tissue**

Female C57Bl/6 mice were given 0.033mg bleomycin i.t. (50µl) +/- liposomal clodronate (LC) i.p. (400µl) on days 21, 22 and 23, and culled on day 25. qPCR was performed on lung homogenates . Following circulating monocyte depletion there is (A) a reduction in *TIMP1* gene expression (bleomycin alone vs. bleomycin, B, *plus* LC on days 21, 22 and 23, p=ns), and (B) an increase in *MMP2* gene expression (bleomycin alone vs. bleomycin, B, *plus* LC on days 21, 22 and 23, p=ns). Representative standard curves used in qPCR analysis are shown for (C) *TIMP1* and (D) *MMP2*. n = 4-5 per group. Results are expressed as mean +/- SEM.

*5.4 Discussion*

Having previously shown that lung macrophages are important for the promotion of pulmonary fibrosis, I have now shown using two models that circulating monocytes are also important for the development of pulmonary fibrosis. I have identified the Ly6C$^{hi}$ monocyte as the cell responsible for this effect, and I have data to suggest that these Ly6C$^{hi}$ monocytes are the precursors of the alternatively activated lung macrophages.

As discussed previously, there has some been interest in the role of tissue macrophages in the pathogenesis of pulmonary fibrosis, but there has been little interest in the role of circulating monocytes. I have already mentioned that there have been two very recent reports suggesting the importance of circulating monocytes in the pathogenesis of pulmonary fibrosis, specifically in relation to IPF (Murray *et al*., 2010) and systemic sclerosis-related interstitial lung disease (Mathai *et al*., 2010).

I have demonstrated that circulating monocytes are important for the development of pulmonary fibrosis and that their depletion leads to a reduction in the proportion of lung and BALF macrophages. This suggests that during pulmonary fibrosis circulating monocytes may be the precursors of interstitial lung and BALF macrophages. This is not surprising as it has been shown that circulating monocytes can be the precursors of lung and BALF macrophages (Landsman & Jung 2007). This however is the first descriptive evidence that in the context of (bleomycin-induced) pulmonary fibrosis, this phenomenon may occur. Furthermore, I go on to show that depletion of circulating monocytes leads to a reduction in gene expression and protein quantity of markers of alternative macrophage activation. This indirectly implicates circulating monocytes as the precursors of alternatively activated lung macrophages, and ties in with our findings discussed in chapter 4. Moreover, I have shown in chapter 4 that intra-tracheal instillation of alternatively activated bone marrow-derived macrophages subsequent to lung macrophage depletion did not restore the fibrotic phenotype. This may reflect the fact that bone marrow-derived macrophages cannot replicate the effects of *in situ*

alternatively activated lung macrophages, or it might add further evidence to our suggestion that alternatively activated lung macrophages are indeed descended from circulating monocytes.

Fibrocytes have been identified in the blood of mice and humans developing pulmonary fibrosis and have been implicated in their pathogenesis (Phillips *et al*., 2004; Andersson-Sjöland *et al*., 2008; Mehrad *et al*., 2009; Moeller *et al*., 2009). Furthermore, it has recently been demonstrated in mice that fibrocytes develop from $CD115^{+}CD11b^{+}Ly6C^{hi}$ monocytes (Niedermeier *et al.,* 2009). This monocyte population is equivalent to the $CD115^{+}CD11b^{+}7/4^{hi}$ monocyte cell population that was depleted by liposomal clodronate in our experiments. It is also identical to the monocyte population of cells that I adoptively transferred into bleomycin-exposed mice with a consequent worsening of pulmonary fibrosis. I have shown that CX3CR1-null mice do not display a difference in pulmonary fibrosis compared to wild-type. $Ly6C^{hi}$ monocytes are $CX3CR1^{lo}$, and so this data complements our results implicating the $Ly6C^{hi}$ monocyte as the promoter of pulmonary fibrosis. Furthermore, it has been recently been published that this $Ly6C^{hi}$ ($CD14^{+}CD16^{-}$ in humans) population of monocytes exhibit up-regulation of markers of alternative macrophage activation, Ym1 (chitinase 3-like protein 3) in mice and CD163 in humans (Ingersoll *et al*., 2010). My experiments show that depletion of $Ly6C^{hi}$ monocytes reduces the numbers of Ym1 positive macrophages in lung. Moreover, in chapter 4 I demonstrated that CD163 is up-regulated on BALF macrophages from patients with IPF, but not in elderly healthy volunteers. I may have therefore provided the first link between fibrocytes, $Ly6C^{hi}$ monocytes, alternatively activated macrophages and pulmonary fibrosis. It is interesting to note as previously discussed that corticosteroids actually induce the alternatively activated phenotype (Gratchev *et al*., 2001; Gratchev *et al*., 2005), and this in part would explain the resistance of patients with IPF to corticosteroid therapy. Our findings therefore bring together a whole host of experimental data from different sources, complemented by our own original data that hopefully clears up one of the long unanswered questions and highly contentious areas in pulmonary fibrosis research.

It is interesting to note that I demonstrated that depletion of circulating monocytes in the CD11b-DTR transgenic mouse did not result in reduced pulmonary fibrosis. This may be explained by 2 factors. Firstly, having worked with this transgenic mouse it is clear that depletion of monocytes may have significant effects on other tissues of the body, such as the monocytes patrolling the gastro-intestinal mucosa. These mice become increasingly unwell as time progresses post-DT administration, and this may confound any effects produced by depletion of circulating monocytes *per se*, particularly where mice survive for many days beyond DT administration, as was the case in our studies. Secondly, recent unpublished work from our own group (Prakash Ramachandran, personal communication) appears to show that DT predominantly and preferentially depletes $Ly6C^{lo}$ monocytes. As such this would instantly explain why no reduction in fibrosis was seen using the CD11b-DTR mouse.

I went on to examine the effects of depletion of circulating monocytes on *MMP/TIMP* gene expression within lung tissue. Depletion of circulating monocytes lead to a reduction in *TIMP1* gene expression and an increase in *MMP2* gene expression. These results are in keeping with those demonstrated by lung macrophage depletion, as highlighted in chapter 4, where depletion of monocytes/macrophages produces an MMP/TIMP ratio that favours lung tissue matrix degradation.

In summary, I have shown the importance of circulating monocytes in the pathogenesis of pulmonary fibrosis. I have identified the $Ly6C^{hi}$ monocyte as the promoter of pulmonary fibrosis and possibly the precursor of lung alternatively activated macrophages. My data further highlights the importance of MMPs and their inhibitor, TIMP1, to the promotion and resolution of pulmonary fibrosis. Finally, and most importantly, having provided a possible link between fibrocytes, $Ly6C^{hi}$ monocytes, alternatively activated lung macrophages, pulmonary fibrosis and steroid resistance, I hope to have informed greater understanding of what remains a devastating and untreatable condition. By doing so, I would hope that new targets are identified,

effective therapies produced, and some hope is brought back into the life of patients with pulmonary fibrosis, and in particular IPF.

# CHAPTER 6

## CONCLUDING REMARKS AND FUTURE STUDIES

During this PhD thesis I have investigated the role of circulating monocytes and lung macrophages in the pathogenesis of pulmonary fibrosis. I have demonstrated that they are not important for the inflammation initiation of fibrosis, and that their depletion during the inflammation stage has no effect on consequent pulmonary fibrosis in the bleomycin model. However, I have established that circulating monocytes and lung macrophages are important during the progressive fibrotic phase of lung fibrogenesis, and that lung macrophages have important but opposite effects during lung fibrosis resolution. I hypothesised that pro-fibrotic alternatively activated macrophages may be the key macrophages that promote fibrosis, and I have established that this indeed appears to be the case in mice, and that alternatively activated macrophages characterise the human form of disease. Furthermore, my work has identified that $Ly6C^{hi}$ circulating monocytes may be the precursors of alternatively activated lung macrophages, but even if they are not, they are important in the pathogenesis of lung fibrosis. Finally, this work has suggested that matrix metalloproteinases may have compartmental-specific functions during lung fibrogenesis.

It is important to state that there are obvious limitations of this work. While the bleomycin and AdTGFβ models are undoubtedly the best models in which to study the molecular mechanisms of pulmonary fibrosis, there remains debate as to their direct applicability to the human forms of fibrotic lung disease. While there are pulmonary conditions that undoubtedly progress through an inflammatory phase which may ultimately develop into fibrosis, such as fibrotic NSIP (Kligerman *et al*., 2009) and chronic hypersensitivity pneumonitis (Olson *et al*., 2008), the direct applicability of these models to IPF, *per se*, remains controversial (Gauldie & Kolb 2008).

The methods used to explore the roles of macrophages in pulmonary fibrosis have limitations in themselves. There is no doubt that liposomal clodronate depletes lung macrophages (Thepen *et al*., 1989), and my work has shown that in the context of bleomycin-induced lung fibrosis, macrophages are effectively depleted. However, other off-target effects of liposomal clodronate in the context of bleomycin-induced, and AdTGFβ-induced pulmonary fibrosis are not established and could have confounding effects. The specificity of the CD11c-DTR transgenic mouse in depleting macrophages has potential issues, particularly if one believes that CD11c-positive cells are dendritic cells. However, CD11c is used to "define" both lung macrophages (Gordon & Taylor 2005; van Rijt *et al*., 2005) and lung dendritic cells (Bar-On & Jung 2010), and as lung macrophages are the predominant cell type in the lungs (Landsman & Jung 2007), then it is more likely that the effect of CD11c cellular targetting is depletion of lung macrophages. Importantly, however, I have shown consistent effects of "macrophage" depletion on lung fibrosis in different models, and using different depletional strategies, and it would seem less likely that in each case the outcomes are merely down to off-target effects. In any case, as has been previously mentioned, prominent macrophage biologists have suggested that dendritic cells and macrophages are in fact part of the same group of mononuclear phagocytic cells (Hume 2008), and that the distinctions are more blurred than has been previously been proposed. What may become more important is not to argue about whether the effects are manifested by CD11c-positive macrophages *versus* CD11c-positive dendritic cells, but what subtype of CD11c-positive mononuclear phagocyte is important.

I believe this work has expanded the knowledge and understanding of pulmonary fibrosis. By identifying new key cellular players, it may enable new treatment strategies to be developed, either in the form of drugs or cellular manipulation. However, it has also generated many interesting and exciting questions. Importantly, I show that it is the modulation of "immune-type" cells, monocytes and macrophages, during fibrogenesis that effect the ultimate end-point of fibrosis. While criticisms might remain about the applicability of models that have an inflammatory component, the potential strength of

this work is that the effects are manifested during the post-inflammatory *fibrogenic* phase, and this does have potential translatability to the human diseases, such as IPF, if inflammation (in its broadest sense) plays only a minor role in its disease pathogenesis. This then begs the question as to whether we should talk about inflammation, or whether we should be more specific and talk about "immune-type cells". I believe that this is where the potential translation of my work may exist. Maybe we should be discussing the benefit of "immune-type" drugs that have *specific* cellular targets during particular phases of pulmonary fibrosis, rather than broadly discussing the benefits of generic "anti-inflammatory" drugs *versus* generic anti-fibrotic drugs. I would suggest that my work informs the need to be clear in the forms of therapy that we use in treating "fibrotic" lung diseases, and that we need to be clear at what stages of disease we use particular generic anti-inflammatory drugs, specific "immune-type" cell modulatory drugs, and specific anti-fibrotic drugs. My work raises the potential disheartening issue that steroids could have detrimental effects during fibrotic lung disease. If steroids do in fact induce a pro-fibrotic alternative activation state in monocytes and macrophages, then it is conceivable that steroids will hasten any natural switch in monocytes/macrophages to a more pro-fibrotic phenotype, to the detriment of the patient. At the practical level, clinicians still use steroids in fibrotic lung disease in an attempt to "eliminate" any underlying additional inflammation. I think this raises several questions:

1.) Should we continue to use steroids to dampen any pre-existing inflammation, while recognising and accepting that they may hasten fibrogenesis, and that therefore we need to use an additional "protective" agent in addition to an anti-fibrotic agent?
2.) Should we stop using steroids, but instead use more specific drugs which have specific effects on particular "immune-type" cells but no fibrosis-enhancing effects, in addition to an anti-fibrotic agent?

3.) How many drugs should we be using? Should we adopt an "oncogenic" approach and use several drugs aimed at targeting the disease process at many levels?
4.) Should we be phenotyping patients more rigorously so that we know what stage of their disease they are at so that specific drugs may be used best tailored to the stage of the disease, with the best predicted response to therapy and outcome?

These are but a few questions that can be asked. There are likely to be many more.

In addition, it remains to be established how circulating monocytes and lung macrophages exert their effects. I believe it will be important to perform *in vitro* co-culture experiments to identify the interactions between macrophages and epithelial cells or fibroblasts/myofibroblasts, what soluble factors may enhance these interactions, and what, if any, signalling pathways direct the changes in cellular behaviour? Many strategies could be employed to tease out the answers. My supervisors are in the process of directing research into the interactions between macrophages and epithelial cells; the effect macrophage activation state has on the process, the importance of cell contact in these interactions, and what changes occur at the protein and RNA levels in each of the different cells? A combination of targeted and broader-based interrogation techniques will likely be needed to gain some mechanistic insights.

I have demonstrated that Ly6C$^{hi}$ monocytes are important in the pathogenesis of pulmonary fibrosis and suggested that they may be the precursors of alternatively activated lung macrophages. Together with my supervisors we have instigated a series of experiments to ascertain whether this is the case. By adoptively transferring bone marrow-derived Ly6C$^{hi}$-sorted monocytes from CD45.1 mice to CD45.2 mice treated with intra-tracheal bleomycin, we should be able to identify whether this hypothesis is true, and the manner in which this occurs; does it occur through a Ly6C$^{lo}$ intermediate or not?

We don't know whether the Ly6C$^{hi}$ monocytes facilitate the switch of resident lung macrophages to the alternative activation macrophage phenotype, or whether they engraft and become these cells. This is important therapeutically. If we can target these influxing cells at the level of the blood then any therapy may be ostensibly easier. If however the circulating monocytes facilitate a switch within resident lung macrophages then therapies that prevent Ly6C$^{hi}$ infiltration to lung *and* effect alternatively activated lung macrophages will likely be more difficult. It is important that these possibilities are explored, and that knowledge is informed, so that appropriate and effective therapies are designed with potential efficacy.

Preliminary work with collaborators has suggested that CD204$^{+}$ (macrophage scavenger receptor-1$^{+}$) circulating monocytes may be up-regulated during pulmonary fibrosis in humans. It has recently been shown that CD163$^{+}$ monocytes are up-regulated in blood from patients with IPF (Murray *et al*., 2010), and my own work has shown that CD163 characterises lung macrophages isolated from BALF of patients with IPF. By further defining and phenotyping the circulating monocytes that are important during lung fibrogenesis, we may be better able to target important cell populations, and in the future even enable their manipulation. Should we target them by blocking/depletion/facilitate apoptosis, or should we attempt to induce a switch away from pro-fibrotic. These are some of the potential options that exist. With further research, more necessary knowledge may be obtained, and inform directed therapeutic approaches. It is possible that expansive and difficult experiments will need to be performed before this potential promise becomes a real possibility.

My research has produced very preliminary data to suggest that matrix metalloproteinases may exhibit compartmental-specific functions during lung fibrogenesis. Tissue-specific targeted approaches using a combination of molecular inhibitors or conditional knockouts may further advance understanding. The potential differential effects of MMP2 and MMP9 needs to be investigated. If my initial observations are true, do they reflect changes in secreted or cell-membrane bound

molecules? If MMP9 does facilitate alveolar re-epithelialisation, how does it do so and what cells does it signal to?; is it the case that type II alveolar epithelial cells facilitate this renewal, or is it due to other local, nearby or circulating progenitor cells? All of these questions are important and many approaches to answer these questions could be used. It is important to highlight the limitations of the MMP/TIMP data. This has already been touched on in previous chapters. I have suggested that in the broad context of MMP/TIMP behaviour within the lung *versus* the alveolar space that compartmental differences may exist. The data to suggest this is very provisional and needs firming up with further experiments. The potential sources of the various MMPs/TIMP have not been explored. Is it epithelia, macrophages, or possibly polymorphonuclear cells, such as neutrophils? Not only from an understanding, but from a potential therapeutic point of view, the sources of the described MMPs and TIMP are important. A previous study evaluated the clinical behaviour and survival of patients with IPF (Selman *et al*., 2007). Patients were classified as "'rapid" or "'slow" progressors according to the duration of symptoms before diagnosis. The "rapid" progressors did not have more BALF neutrophils or eosinophils than slow progressor, however, BALF from "rapid" progressors showed a 2-fold increase of active MMP9. The possible role of neutrophils, eosinophils and other polymorphonuclear cells has not been addressed or discussed. It is known for example that neutrophils (Veeraraghavan *et al*., 2003; Kinder *et al*., 2008) and eosinophils (Veeraraghavan *et al*., 2003) are increased in the BALF of patients with IPF. The significance of this is unknown. I have alluded to potential roles of other "immune-type" cells throughout the thesis; their specific roles and effects were not the subject of my thesis, and thus were not explored. However it is clearly an area where research needs to be performed before a comprehensive understanding of the pathogenesis of fibrotic lung disease can be realised.

Finally, my finding that lung macrophages facilitate resolution of lung fibrosis during the reversible phase is a tantalising one. It leaves many unanswered questions. It remains to be determined what phenotype of macrophage produces this effect. Are they produced from local populations or are they recruited from the circulation? What

interactions do they have and what is their mechanism of action? Can we manipulate these "good" macrophages for positive effect and do they have a human counterpart? A limitation of this finding is that in humans fibrosis does not appear to reverse, so where is the direct translatability? Inflammation and organising pneumonia can reverse, but they are not indicative of fibrosis *per se*. As a result, there will undoubtedly be a group of people who believe that my finding of macrophages regulating the reversible phase of fibrosis as of no direct applicability to humans. Strictly speaking this is true. However, if we can gain some mechanistic insight inot how macrophages may help facilitate fibrosis resolution in the mouse, then we may be able to design and produce therapies that can be studied in humans. It may produce real promise. There is no doubt that any translatability to humans is a long way off, but I believe that this is an area where research should be taken forward.

In summary, my research has produced some answers and generated many questions. I believe my research has enabled a better understanding of a devastating disease and I hope new efficacious therapies may be developed so that patients with fibrotic lung diseases have a better future.

## REFERENCES


Abe R, Donnelly SC, Peng T, Bucala R, Metz CN: Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol*. 2001;166:7556–62.

Abramson SL, Gallin JI. IL-4 inhibits superoxide production by human mononuclear phagocytes. *J Immunol*. 1990;144(2):625-30.

Adams DO. Molecular interactions in macrophage activation. *Immunol Today*. 1989;Feb;10(2):33-5.

Adamson IY. Pulmonary toxicity of bleomycin. *.Environ Health Perspect*. 1976;16:119-26.

Adamson IY, Bowden DH. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol*. 1974;77(2):185-97.

Addrizzo-Harris DJ, Harkin TJ, Tchou-Wong KM, McGuinness G, Goldring R, Cheng D, Rom DW. Mechanisms of colchicine effect in the treatment of asbestosis and idiopathic pulmonary fibrosis. *Lung* 2002;180:61–72.

Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and1/4-length fragments. *J Biol Chem* 1995;270:5872–5876.

Akira M, Hamada H, Sakatani M, Kobayashi C, Nishioka M, Yamamoto S. CT findings during phase of accelerated deterioration in patients with idiopathic pulmonary fibrosis. *AJR Am J Roentgenol*. 1997;168(1):79-83.

Ambrosini V, Cancellieri A, Chilosi M, Zompatori M, Trisolini R, Saragoni L, Poletti V. Acute exacerbation of idiopathic pulmonary fibrosis: report of a series. *Eur Respir J* 2003;22:821–826.

American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement. American Thoracic Society (ATS), European Respiratory Society (ERS). *Am J Respir Crit Care Med* 2000;161:646-664.

American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med*. 2002;165:277-304.

Anderson KM, Harris JE, Bonomi P. Potential applications of apoptosis in modifying the biological behavior of therapeutically refractory cancers. *Med Hypotheses*. 1994;43(4):207-13.

Anderson CF, Mosser DM. A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J Leukoc Biol*. 2002;72(1):101-6.

Andersson-Sjöland A, de Alba CG, Nihlberg K, Becerril C, Ramírez R, Pardo A, Westergren-Thorsson G, Selman M. Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis. *Int J Biochem Cell Biol*. 2008;40(10):2129-40.

Arciniegas E, Neves CY, Carrillo LM, Zambrano EA, Ramírez R. Endothelial-mesenchymal transition occurs during embryonic pulmonary artery development. *Endothelium*. 2005;12(4):193-200.

Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol*. 1988;41(4):467-70.

Ask K, Bonniaud P, Maass K, Eickelberg O, Margetts PJ, Warburton D, Groffen J, Gauldie J, Kolb M. Progressive pulmonary fibrosis is mediated by TGF-beta isoform 1 but not TGF-beta3. *Int J Biochem Cell Biol*. 2008;40(3):484-95.

Atabai K, Jame S, Azhar N, Kuo A, Lam M, McKleroy W, Dehart G, Rahman S, Xia DD, Melton AC, Wolters P, Emson CL, Turner SM, Werb Z, Sheppard D. Mfge8 diminishes the severity of tissue fibrosis in mice by binding and targeting collagen for uptake by macrophages. *J Clin Invest*. 2009;119(12):3713-3722.

Atamas SP, Luzina IG, Choi J, Tsymbalyuk N, Carbonetti NH, Singh IS, Trojanowska M, Jimenez SA, White B. Pulmonary and activation-regulated chemokine stimulates collagen production in lung fibroblasts. *Am J Respir Cell Mol Biol* 2003;29:743-7.

Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol.* 2003;28(1):12-24.

Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007 Aug 3;317(5838):666-70.

Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol.* 2009;27:669-92.

Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 1981;11(10):805-15.

Azuma A, Nukiwa T, Tsuboi E, Suga M, Abe S, Nakata K, Taguchi Y, Nagai S, Itoh H, Ohi M, et al. Placebo-controlled trial of Pirfenidone in patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2005;171:1040–1047.

Bar-On L, Jung S. Defining in vivo dendritic cell functions using CD11c-DTR transgenic mice. *Methods Mol Biol*. 2010;595:429-42.

Baran CP, Opalek JM, McMaken S, Newland CA, O'Brien JM Jr, Hunter MG, Bringardner BD, Monick MM, Brigstock DR, Stromberg PC, Hunninghake GW, Marsh CB. Important roles for macrophage colony-stimulating factor, CC chemokine ligand 2, and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis. *Am J Respir Crit Care Med*. 2007;176(1):78-89.

Barbarin V, Nihoul A, Misson P, Arras M, Delos M, Leclercq I, Lison D, Huaux F. The role of pro- and anti-inflammatory responses in silica-induced lung fibrosis. *Respir Res*. 2005;6:112.

Bedrossian CW, Greenberg SD, Yawn DH, O'Neal RM. Experimentally induced bleomycin sulfate pulmonary toxicity: histopathologic and ultrastructural study in the pheasant. *Arch Pathol Lab Med*. 1977;101(5):248-54.

Bem RA, Farnand AW, Wong V, Koski A, Rosenfeld ME, van Rooijen N, Frevert CW, Martin TR, Matute-Bello G. Depletion of resident alveolar macrophages does not prevent Fas-mediated lung injury in mice. *Am J Physiol Lung Cell Mol Physiol*. 2008;295(2):L314-325.

Betsuyaku T, Fukuda Y, Parks WC, Shipley JM, Senior RM. Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. *Am J Pathol*. 2000;157(2):525-35.

Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci U S A*. 1994;91(19):8802-8806.

Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, Bottazzi B, Doni A, Vincenzo B, Pasqualini F, Vago L, Nebuloni M, Mantovani A, Sica A. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood*. 2006;107(5):2112-22.

Bitterman PB, Adelberg S, Crystal RG. Mechanisms of pulmonary fibrosis. Spontaneous release of the alveolar macrophage-derived growth factor in the interstitial lung disorders. *J Clin Invest*. 1983;72(5):1801-1813.

Bitterman PB, Saltzman LE, Adelberg S, Ferrans VJ, Crystal RG. Alveolar macrophage replication. One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. *J Clin Invest*. 1984;74(2):460-469.

Bitterman PB, Wewers MD, Rennard SI, Adelberg S, Crystal RG. Modulation of alveolar macrophage-driven fibroblast proliferation by alternative macrophage mediators. *J Clin Invest*. 1986;77(3):700-708.

Bjoraker JA, Ryu JH, Edwin MK, Myers JL, Tazelaar HD, Schroeder DR, Offord KP. Prognostic significance of histopathologic subsets in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1998; 157(1): 199-203.

Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol*. 1997;15:749-95.

Bogdan C, Paik J, Vodovotz Y, Nathan C. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *J Biol Chem*. 1992;267(32):23301-8.

Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, Chantry D, Mantovani A. Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood*. 1998;92(8):2668-71.

Bonner JC, Lindroos PM, Rice AB, Moomaw CR, Morgan DL. Induction of PDGF receptor-alpha in rat myofibroblasts during pulmonary fibrogenesis in vivo. *Am J Physiol.* 1998;274(1 Pt 1):L72-80.

Bonniaud P, Margetts PJ, Kolb M, Haberberger T, Kelly M, Robertson J, Gauldie J. Adenoviral gene transfer of connective tissue growth factor in the lung induces transient fibrosis. *Am J Respir Crit Care Med*. 2003;168(7):770-8.

Bonniaud P, Martin G, Margetts PJ, Ask K, Robertson J, Gauldie J, Kolb M. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. *Am J Respir Cell Mol Biol*. 2004;31(5):510-6.

Borzone G, Moreno R, Urrea R, Meneses M, Oyarzún M, Lisboa C. Bleomycin-induced chronic lung damage does not resemble human idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2001;163(7):1648-53.

Bouhlel MA, Derudas B, Rigamonti E, Dièvart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab*. 2007;6(2):137-43.

Bowden DH, Adamson IY, Grantham WG, Wyatt JP. Origin of the lung macrophage. Evidence derived from radiation injury. *Arch Pathol*. 1969;88(5):540-6.

Bowden DH, Adamson IY. The pulmonary interstitial cell as immediate precursor of the alveolar macrophage. *Am J Pathol*. 1972;68(3):521-37.

Bramson JL, Graham FL, Gauldie J. The use of adenoviral vectors for gene therapy and gene transfer in vivo. *Curr Opin Biotechnol*. 1995;6(5):590-5.

Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor□1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991;88(15):6642–6646.

Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med*. 1994;1(1):71-81.

Budinger GR, Mutlu GM, Eisenbart J, Fuller AC, Bellmeyer AA, Baker CM, Wilson M, Ridge K, Barrett TA, Lee VY, Chandel NS. Proapoptotic Bid is required for pulmonary fibrosis. *Proc Natl Acad Sci U S A*. 2006;103(12):4604-9.

Cabrera S, Gaxiola M, Arreola JL, Ramirez R, Jara P, D'Armiento J, Richards T, Selman M, Pardo A. Overexpression of MMP9 in macrophages attenuates pulmonary fibrosis induced by bleomycin. *Int J Biochem Cell Biol*. 2007;39(12):2324–38.

Cailhier JF, Partolina M, Vuthoori S, Wu S, Ko K, Watson S, Savill J, Hughes J, Lang RA. Conditional macrophage ablation demonstrates that resident macrophages initiate acute peritoneal inflammation. *J Immunol*. 2005;174(4):2336-42.

Cao H, Wolff RG, Meltzer MS, Crawford RM. Differential regulation of class II MHC determinants on macrophages by IFN-gamma and IL-4. *J Immunol*. 1989;143(11):3524-31.

Carlin JM, Borden EC, Byrne GI. Interferon-induced indoleamine 2,3-dioxygenase activity inhibits Chlamydia psittaci replication in human macrophages. *J Interferon Res.* 1989;9(3):329-37.

Chen J, Ghorai MK, Kenney G, Stubbe J. Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage. *Nucleic Acids Res*. 2008;36(11):3781-90.

Chizzolini C, Rezzonico R, De Luca C, Burger D, Dayer JM. Th2 cell membrane factors in association with IL-4 enhance matrix metalloproteinase-1 (MMP-1) while decreasing MMP-9 production by granulocyte-macrophage colony-stimulating factor-differentiated human monocytes. *J Immunol*. 2000;164(11):5952-60.

Choi KH, Lee HB, Jeong MY, Rhee YK, Chung MJ, Kwak YG, Lee YC. The role of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in cryptogenic organizing pneumonia. *Chest*. 2002 May;121(5):1478-85.

Choi JE, Lee SS, Sunde DA, Huizar I, Haugk KL, Thannickal VJ, Vittal R, Plymate SR, Schnapp LM. Insulin-like growth factor-I receptor blockade improves outcome in mouse model of lung injury. *Am J Respir Crit Care Med*. 2009;179(3):212-219.

Christensen PJ, Goodman RE, Pastoriza L, Moore B, Toews GB. Induction of lung fibrosis in the mouse by intratracheal instillation of fluorescein isothiocyanate is not T-cell-dependent. *Am J Pathol*. 1999;155(5):1773-9.

Chua F, Gauldie J, Laurent GJ. Pulmonary fibrosis: searching for model answers. *Am J Respir Cell Mol Biol*. 2005;33(1):9-13.

Chua F, Dunsmore SE, Clingen PH, Mutsaers SE, Shapiro SD, Segal AW, Roes J, Laurent GJ. Mice lacking neutrophil elastase are resistant to bleomycin-induced pulmonary fibrosis. *Am J Pathol*. 2007;170(1):65-74.

Civil GW, Heppleston AG. Replenishment of alveolar macrophages in silicosis: implication of recruitment by lipid feed-back. *Br J Exp Pathol*. 1979;60(5):537-47.

Clozel M, Breu V, Gray GA, Kalina B, Löffler BM, Burri K, Cassal JM, Hirth G, Müller M, Neidhart W, *et al*.. Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J Pharmacol Exp Ther*. 1994;270(1):228-35.

Collard HR, Moore BB, Flaherty KR, Brown KK, Kaner RJ, King TE Jr, Lasky JA, Loyd JE, Noth I, Olman MA, Raghu G, Roman J, Ryu JH, Zisman DA, Hunninghake GW, Colby TV, Egan JJ, Hansell DM, Johkoh T, Kaminski N, Kim DS, Kondoh Y, Lynch DA, Müller-Quernheim J, Myers JL, Nicholson AG, Selman M, Toews GB, Wells AU, Martinez FJ; Idiopathic Pulmonary Fibrosis Clinical Research Network Investigators. Acute exacerbations of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2007;176(7):636-43.

Collard HR. Idiopathic pulmonary fibrosis and pirfenidone. *Eur Respir J*. 2010;35(4):728-9.

Collins T, Ginsburg D, Boss JM, Orkin SH, Pober JS. Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis. *Nature.* 1985;316(6030):748-50.

Cool CD, Groshong SD, Rai PR, Henson PM, Stewart JS, Brown KK. Fibroblast foci are not discrete sites of lung injury or repair: the fibroblast reticulum. *Am J Respir Crit Care Med*. 2006;174(6):654-8.

Cooper JA Jr, White DA and Matthay RA. Drug-induced pulmonary disease 1. Cytotoxic drugs. *Am Rev Respir Dis* 1986;133: 321–40.

Corraliza IM, Campo ML, Soler G, Modolell M. Determination of arginase activity in macrophages: a micromethod. *J Immunol Methods*. 1994; 174(1-2):231–235.

Coultas DB, Zumwalt RE, Black WC and Sobonya RE. The epidemiology of interstitial lung disease. *Am J Respir Crit Care Med.* 1994;150(4):967–72.

de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med.* 1991;174(4):915-24.

du Bois RM & King TE Jr. Clinical advances in the diagnosis and therapy of the interstitial pneumonias. *Thorax.* 2007;62:1008-1012.

du Bois RM. Strategies for treating idiopathic pulmonary fibrosis. *Nat Rev Drug* Discovery. 2010;9(2):129-40.

D'Andrea A, Ma X, Aste-Amezaga M, Paganin C, Trinchieri G. Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production. *J Exp Med*. 1995;181(2):537-46.

Dark JH. Priorities for lung transplantation. *Lancet* 1998; 351(9095): 4-5.

Dauber JH, Rossman MD, Pietra GG, Jimenez SA, Daniele RP. Experimental silicosis: morphologic and biochemical abnormalities produced by intratracheal instillation of quartz into guinea pig lungs. *Am J Pathol*. 1980;101(3):595-612.

Davies HR, Richeldi L, Walters EH. Immunomodulatory agents for idiopathic pulmonary fibrosis. Cochrane Database Syst Rev 2003; issue 2:CD003134.

Davis GS, Leslie KO, Hemenway DR. Silicosis in mice: effects of dose, time, and genetic strain. *J Environ Pathol Toxicol Oncol*. 1998;17(2):81-97.

a. Desmoulière A, Rubbia-Brandt L, Abdiu A, Walz T, Macieira-Coelho A, Gabbiani G. Alpha-smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by gamma-interferon. *Exp Cell Res*. 1992;201(1):64-73.

b. Desmoulière A, Rubbia-Brandt L, Grau G, Gabbiani G. Heparin induces alpha-smooth muscle actin expression in cultured fibroblasts and in granulation tissue myofibroblasts. *Lab Invest*. 1992;67(6):716-26.

Dockrell DH, Marriott HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, Whyte MK. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol*. 2003;171(10):5380-8.

Douglas WW, Ryu JH, Bjoraker JA, Schroeder DR, Myers JL, Tazelaar HD, Swensen SJ, Scanlon PD, Peters SG, DeRemee RA. Colchicine versus prednisone as treatment of usual interstitial pneumonia. *Mayo Clin Proc* 1997;72:201–209.

Douglas WW, Ryu JH, Swensen SJ, Offord KP, Schroeder DR, Caron GM, DeRemee RA. Colchicine versus prednisone in the treatment of idiopathic pulmonary fibrosis: a

randomized prospective study. Members of the Lung Study Group. *Am J Respir Crit Care Med* 1998; 158:220–225.

Douglas WW, Ryu JH, Schroeder DR. Idiopathic pulmonary fibrosis: Impact of oxygen and colchicine, prednisone, or no therapy on survival. *Am J Respir Crit Care Med* 2000;161:1172–1178.

a. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115(1):56-65.

b. Duffield JS, Tipping PG, Kipari T, Cailhier JF, Clay S, Lang R, Bonventre JV, Hughes J. Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *Am J Pathol*. 2005;167(5):1207-19.

Ehrchen J, Steinmüller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M, Nordhues U, Sorg C, Sunderkötter C, Roth J. Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood*. 2007;109(3):1265-74.

Fallowfield JA, Mizuno M, Kendall TJ, Constandinou CM, Benyon RC, Duffield JS, Iredale JP. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol*. 2007;178(8):5288-95.

Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med*. 2006;12(1):99-106.

Fisher CE, Ahmad SA, Fitch PM, Lamb JR, Howie SE. FITC-induced murine pulmonary inflammation: CC10 up-regulation and concurrent Shh expression. *Cell Biol Int.* 2005;29(10):868-76.

a. Flaherty KR, Toews GB, Lynch JP III, Kazerooni EA, Gross BH, Strawderman RL III, Hariharan K, Flint A, Martinez FJ. Steroids in idiopathic pulmonary fibrosis: a prospective assessment of adverse reactions, response to therapy, and survival. *Am J Med* 2001;110:278– 282.

b. Flaherty KR, Travis WD, Colby TV, Toews GB, Kazerooni EA, Gross BH, Jain A, Strawderman RL, Flint A, Lynch JP, Martinez FJ. Histopathologic variability in usual and non-specific interstitial pneumonias. *Am J Respir Crit Care Med.* 2001;164(9):1722-7.

Fleischman RW, Baker JR, Thompson GR, Schaeppi UH, Illievski VR, Cooney DA, Davis RD. Bleomycin-induced interstitial pneumonia in dogs. *Thorax*. 1971;26(6):675-82.

Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol*. 1993;151(5):2399-408.

Franko AJ, Sharplin J, Ward WF, Taylor JM. Evidence for two patterns of inheritance of sensitivity to induction of lung fibrosis in mice by radiation, one of which involves two genes. *Radiat Res*. 1996;146(1):68-74.

Fulmer J, Elson N, Von Gal E, McLees B, MeMets D, Weinberger S, Kelman J, Crystal RG, *et al*. Treatment of idiopathic pulmonary fibrosis [abstract]. *Clin Res* 1978;26:538A.

Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia*. 1971;27(5):549-50.

Gauldie J. Pro: Inflammatory mechanisms are a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2002; 165(9):1205-6.

Gauldie J, Kolb M. Animal models of pulmonary fibrosis: how far from effective reality? *Am J Physiol Lung Cell Mol Physiol*. 2008;294(2):L151.

Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19(1):71-82.

Getts DR, Terry RL, Getts MT, Müller M, Rana S, Shrestha B, Radford J, Van Rooijen N, Campbell IL, King NJ. Ly6C$^{+}$ "inflammatory monocytes" are microglial precursors recruited in a pathogenic manner in West Nile virus encephalitis. *J Exp Med*. 2008;205(10):2319-2337.

Gharaee-Kermani M, Hu B, Phan SH, Gyetko MR. Recent advances in molecular targets and treatment of idiopathic pulmonary fibrosis: focus on TGFbeta signaling and the myofibroblast. *Curr Med Chem*. 2009;16(11):1400-17.

Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. *Nat Rev Drug Discov*. 2004;3(5):401-16.

Giri SN, Hyde DM, Hollinger MA. Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice. *Thorax*. 1993;48(10):959-66.

Giri SN, Hyde DM, Braun RK, Gaarde W, Harper JR, Pierschbacher MD. Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. *Biochem Pharmacol* 1997;54(11):1205–1216.

Goldstein B, Webster I. Intratracheal injection into rats of size-graded silica particles. *Br J Ind Med*. 1966;23(1):71-4.

Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3(1):23-35.

Gordon S and Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 2005;5(12):953-64.

Gordon S. Macrophage heterogeneity and tissue lipids. *J Clin Invest*. 2007;117(1): 89-93.

Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*. 1977;36(1):59-74.

Gratchev A, Guillot P, Hakiy N, Politz O, Orfanos CE, Schledzewski K, Goerdt S. Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. *Scand J Immunol*. 2001;53(4):386-392.

Gratchev A, Kzhyshkowska J, Utikal J, Goerdt S. Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. *Scand J Immunol*. 2005 ;61(1):10-17.

Gross TJ, Hunninghake, GW. Idiopathic pulmonary fibrosis. *N Engl J Med*. 2001;345: 517–525.

Gruenheid S, Gros P. Genetic susceptibility to intracellular infections: Nramp1, macrophage function and divalent cations transport. *Curr Opin Microbiol*. 2000;3(1):43-8.

Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal.* 2001;13(2):85-94.

Gurujeyalakshmi G, Hollinger MA, Giri SN. Pirfenidone inhibits PDGF isoforms in bleomycin hamster model of lung fibrosis at the translational level. *Am J Physiol*. 1999;276(2 Pt 1):L311-8.

Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, Hara N. Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Cell Mol Biol*. 1997;16(1):91-101.

Hancock A, Armstrong L, Gama R, Millar A. Production of interleukin 13 by alveolar macrophages from normal and fibrotic lung. *Am J Respir Cell Mol Biol*. 1998;18(1):60-5.

a. Hart PH, Burgess DR, Vitti GF, Hamilton JA. Interleukin-4 stimulates human monocytes to produce tissue-type plasminogen activator. *Blood*. 1989;74(4):1222-5.

b. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci U S A*. 1989;86(10):3803-7.

Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH. Bone marrow derived progenitor cells in pulmonary fibrosis. *J Clin Invest*. 2004;113:243–252.

Hashimoto N, Phan SH, Imaizumi K, Matsuo M, Nakashima H, Kawabe T, Shimokata K, Hasegawa Y. Endothelial-mesenchymal transition in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2009;Sep 18. [Epub ahead of print].

Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med*. 1999;160:S5–S11.

Haston CK, Travis EL. Murine susceptibility to radiation-induced pulmonary fibrosis is influenced by a genetic factor implicated in susceptibility to bleomycin-induced pulmonary fibrosis. *Cancer Res*. 1997;57(23):5286-91.

Hatch GE, Raub JA, Graham JA. Functional and biochemical indicators of pneumoconiosis in mice: comparison with rats. *J Toxicol Environ Health*. 1984;13(4-6):487-97.

Henderson NC, MacKinnon AC, Farnworth SL, Kipari T, Haslett C, Iredale JP, Liu FT, Hughes J, Sethi T. Galectin-3 expression and secretion links macrophages to the promotion of renal fibrosis. *Am J Pathol*. 2008;172(2):288-298.

Heppleston AG. The fibrogenic action of silica. *Br Med Bull*. 1969;25(3):282-7.

Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, Pearce EJ, Wynn TA. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol*. 2001;167(11):6533-44.

Higashiyama H, Yoshimoto D, Okamoto Y, Kikkawa H, Asano S, Kinoshita M. Receptor-activated Smad localisation in bleomycin-induced pulmonary fibrosis. *J Clin Pathol*. 2007;60(3):283-9.

Hochweller K, Striegler J, Hämmerling GJ, Garbi N. A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells. *Eur J Immunol*. 2008;38(10):2776-2783.

Högger P, Dreier J, Droste A, Buck F, Sorg C. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J Immunol*. 1998;161(4):1883-90.

Holt PG. Down-regulation of immune responses in the lower respiratory tract: the role of alveolar macrophages. *Clin Exp Immunol.* 1986;63(2):261-70.

Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med*. 1993;177(2):397-407.

Homma S, Nagaoka I, Abe H, Takahashi K, Seyama K, Nukiwa T, Kira S. Localization of platelet-derived growth factor and insulin like growth factor I in the fibrotic lung. *Am J Respir Crit Care Med*. 1995;152(6 Pt 1):2084–2089.

Homma S, Sakamoto S, Kawabata M, Kishi K, Tsuboi E, Motoi N, Yoshimura K. Cyclosporin treatment in steroid-resistant and acutely exacerbated interstitial pneumonia. *Intern Med* 2005;44:1144–1150.

Horan GS, Wood S, Ona V, Li DJ, Lukashev ME, Weinreb PH, Simon KJ, Hahm K, Allaire NE, Rinaldi NJ, Goyal J, Feghali-Bostwick CA, Matteson EL, O'Hara C, Lafyatis R, Davis GS, Huang X, Sheppard D, Violette SM. Partial inhibition of integrin alpha(v)beta6 prevents pulmonary fibrosis without exacerbating inflammation. *Am J Respir Crit Care Med.* 2008;177(1):56-65.

Hosenpud JD, Bennett LE, Keck BM, Edwards EB, Novick RJ. Effect of diagnosis on survival benefit of lung transplantation for end-stage lung disease. *Lancet* 1998; 351(9095): 24-27.

Hume DA. Macrophages as APC and the dendritic cell myth. *J Immunol*. 2008;181(9):5829-35.

Hunninghake GW, Lynch DA, Galvin JR, Gross BH, Müller N, Schwartz DA, King TE Jr, Lynch JP 3rd, Hegele R, Waldron J, Colby TV, Hogg JC. Radiologic findings are strongly associated with a pathologic diagnosis of usual interstitial pneumonia. *Chest.* 2003;124(4):1215-23.

Hunter M, Wang Y, Eubank T, Baran C, Nana-Sinkam P, Marsh C. Survival of monocytes and macrophages and their role in health and disease. *Front Biosci*. 2009;14:4079-102.

Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, Lang R, Haniffa M, Collin M, Tacke F, Habenicht AJ, Ziegler-Heitbrock L, Randolph GJ. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood.* 2010;115(3):e10-9.

Ishii M, Wen H, Corsa CA, Liu T, Coelho AL, Allen RM, Carson WF 4th, Cavassani KA, Li X, Lukacs NW, Hogaboam CM, Dou Y, Kunkel SL. Epigenetic regulation of the alternatively activated macrophage phenotype. *Blood.* 2009;114(15):3244-54.

Iyer SN, Wild JS, Schiedt MJ, Hyde DM, Margolin SB, Giri SN. Dietary intake of pirfenidone ameliorates bleomycin-induced lung fibrosis in hamsters. *J Lab Clin Med*. 1995;125(6):779-85.

Iyer SN, Margolin SB, Hyde DM, Giri SN. Lung fibrosis is ameliorated by pirfenidone fed in diet after the second dose in a three-dose bleomycin-hamster model. *Exp Lung Res*. 1998;24(1):119-32.

a. Iyer SN, Gurujeyalakshmi G, Giri SN. Effects of pirfenidone on procollagen gene expression at the transcriptional level in bleomycin hamster model of lung fibrosis. *J Pharmacol Exp Ther.* 1999;289(1):211-8.

b. Iyer SN, Gurujeyalakshmi G, Giri SN. Effects of pirfenidone on transforming growth factor-beta gene expression at the transcriptional level in bleomycin hamster model of lung fibrosis. *J Pharmacol Exp Ther*. 1999;291(1):367-73.

Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. *Int J Exp Pathol*. 2002;83(3):111-119.

Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, Traver D, van Rooijen N, Weissman IL. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell.* 2009;138(2):271-285.

Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.

Johansen JS, Møller S, Price PA, Bendtsen F, Junge J, Garbarsch C, Henriksen JH. Plasma YKL-40: a new potential marker of fibrosis in patients with alcoholic cirrhosis? *Scand J Gastroenterol*. 1997;32(6):582-90.

Johansen JS, Christoffersen P, Møller S, Price PA, Henriksen JH, Garbarsch C, Bendtsen F. Serum YKL-40 is increased in patients with hepatic fibrosis. *J Hepatol*. 2000;32(6):911-20.

Johnson MA, Kwan S, Snell NJC, Nunn AJ, Darbyshire JH, Turner- Warwick M. Randomized controlled trial comparing prednisolone alone with cyclophosphamide and low dose prednisolone in combination in cryptogenic fibrosing alveolitis. *Thorax* 1989;44:280–288.

Johnston CJ, Wright TW, Rubin P, Finkelstein JN. Alterations in the expression of chemokine mRNA levels in fibrosis-resistant and -sensitive mice after thoracic irradiation. *Exp Lung Res*. 1998;24(3):321-37.

Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, Littman DR Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol*. 2000 Jun;20(11):4106-14.

Kahnert A, Seiler P, Stein M, Bandermann S, Hahnke K, Mollenkopf H, Kaufmann SH. Alternative activation deprives macrophages of a coordinated defense program to Mycobacterium tuberculosis. *Eur J Immunol*. 2006;36(3):631-47.

Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, Merad M, Luedde T, Trautwein C, Tacke F. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology.* 2009;50(1):261-74.

Katzenstein AL and Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathological classification. *Am J Respir Crit Care Med*. 1998;157(4 Pt 1):1301-15.

Keane MP. The role of chemokines and cytokines in lung fibrosis. *European Respiratory Review*. 2008;17:151-6.

Khalil N, Bereznay O, Sporn M, Greenberg AH. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med*. 1989;170(3):727-737.

Khalil N, Whitman C, Zuo L, Danielpour D, Greenberg A. Regulation of alveolar macrophage transforming growth factor-beta secretion by corticosteroids in bleomycin-induced pulmonary inflammation in the rat. *J Clin Invest*. 1993;92(4):1812-1818.

Khalil N and O'Connor R. Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment. *CMAJ*. 2004;171:153-60.

Kim DS, Park JH, Park BK, Lee JS, Nicholson AG, Colby T. Acute exacerbation of idiopathic pulmonary fibrosis: frequency and clinical features. *Eur Respir J* 2006;27:143–150.

Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci USA*. 2006;103:13180–5.

Kinder BW, Brown KK, Schwarz MI, Ix JH, Kervitsky A, King TE Jr. Baseline BAL neutrophilia predicts early mortality in idiopathic pulmonary fibrosis. *Chest*. 2008;133(1):226-32.

King EJ, Mohanty GP, Harrison CV, Nagelschmidt G. The action of flint of variable size injected at constant weight and constant surface into the lungs of rats. *Br J Ind Med.* 1953;10(2):76-92.

King TE Jr, Costabel U, Cordier J-F, doPico GA, du Bois RM, Lynch D, Lynch JP III, Myers JL, Panos RJ, Raghu G, Schwartz D, Smith CM. Idiopathic pulmonary fibrosis:

diagnosis and treatment. International Consensus Statement. *Am J Respir Crit Care Med*. 2000;161:646–64.

King TE Jr, Schwarz MI, Brown K et al. Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. *Am J Respir Crit Care Med*. 2001; 164(6):1025-32.

King TE Jr, Behr J, Brown KK, du Bois RM, Lancaster L, de Andrade JA, Stähler G, Leconte I, Roux S, Raghu G. BUILD-1: a randomized placebo-controlled trial of bosentan in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2008;177(1):75-81.

King TE Jr, Albera C, Bradford WZ, Costabel U, Hormel P, Lancaster L, Noble PW, Sahn SA, Szwarcberg J, Thomeer M, Valeyre D, du Bois RM; INSPIRE Study Group. Effect of interferon gamma-1b on survival in patients with idiopathic pulmonary fibrosis (INSPIRE): a multicentre, randomised, placebo-controlled trial. *Lancet.* 2009;374(9685):222-8

Kirby AC, Raynes JG, Kaye PM. CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge. *J Infect Dis*. 2006;193(2):205-13.

Kirk JME, Heard BE, Kerr I, Turner-Warwick M, Laurent GJ. Quantitation of types I and III collagen in biopsy lung samples from patients with cryptogenic fibrosing alveolitis. *Collagen Rel Res* 1984;4:169–182.

Kligerman SJ, Groshong S, Brown KK, Lynch DA. Nonspecific interstitial pneumonia: radiologic, clinical, and pathologic considerations. *Radiographics*. 2009;29(1):73-87.

Kodelja V, Müller C, Tenorio S, Schebesch C, Orfanos CE, Goerdt S. Differences in angiogenic potential of classically vs alternatively activated macrophages. *Immunobiology*. 1997;197(5):478-93.

Kodelja V, Müller C, Politz O, Hakij N, Orfanos CE, Goerdt S. Alternative macrophage activation-associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-1 alpha with a Th2-associated expression pattern. *J Immunol.* 1998;160(3):1411-8.

Kolb M, Margetts PJ, Galt T, Sime PJ, Xing Z, Schmidt M, Gauldie J. Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am J Respir Crit Care Med*. 2001;163(3 Pt 1):770-7.

Kondoh Y, Taniguchi H, Kawabata Y, Yokoi T, Suzuki K, Takagi K. Acute exacerbation in idiopathic pulmonary fibrosis: analysis of clinical and pathologic findings in three cases. *Chest* 1993;103:1808–1812.

Kross J, Henner WD, Hecht SM, Haseltine WA. Specificity of deoxyribonucleic acid cleavage by bleomycin, phleomycin, and tallysomycin. *Biochemistry.* 1982;21(18):4310-8.

Kubo H, Nakayama K, Yanai M, Suzuki T, Yamaya M, Watanabe M, Sasaki H. Anticoagulant therapy for idiopathic pulmonary fibrosis. *Chest* 2005;128:1475–1482.

Kuroda E, Ho V, Ruschmann J, Antignano F, Hamilton M, Rauh MJ, Antov A, Flavell RA, Sly LM, Krystal G. SHIP represses the generation of IL-3-induced M2 macrophages by inhibiting IL-4 production from basophils. *J Immunol*. 2009;183(6):3652-60.

Lacronique JG, Rennard SI, Bitterman PB, Ozaki T, Crystal RG. Alveolar macrophages in idiopathic pulmonary fibrosis have glucocorticoid receptors, but glucocorticoid therapy does not suppress alveolar macrophage release of fibronectin and alveolar macrophage derived growth factor. *Am Rev Respir Dis*. 1984;130(3):450-456.

Lagasse E, Weissman IL. Flow cytometric identification of murine neutrophils and monocytes. *J Immunol Methods*. 1996;197(1-2):139-50.

Lambrecht BN. Alveolar macrophage in the driver's seat. *Immunity*. 2006; 24(4):366-8.

Landsman L and Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol*. 2007;179(6):3488-94.

Landsman L, Varol C and Jung S. Distinct differentiation potential of blood monocyte subsets in the lung. *J Immunol*. 2007;178(4):2000-7.

Lauener RP, Goyert SM, Geha RS, Vercelli D. Interleukin 4 down-regulates the expression of CD14 in normal human monocytes. *Eur J Immunol*. 1990;20(11):2375-81.

Laurent GJ, McAnulty RJ, Corrin B, Cockerill P. Biochemical and histological changes in pulmonary fibrosis induced in rabbits with intratracheal bleomycin. *Eur J Clin Invest*. 1981;11(6):441-8.

Lawson WE, Polosukhin VV, Stathopoulos GT, Zoia O, Han W, Lane KB, Li B, Donnelly EF, Holburn GE, Lewis KG, Collins RD, Hull WM, Glasser SW, Whitsett JA, Blackwell TS. Increased and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. *Am J Pathol*. 2005;167(5):1267-77.

Lee CG, Elias JA. Role of breast regression protein-39/YKL-40 in asthma and allergic responses. *Allergy Asthma Immunol Res*. 2010;2(1):20-7.

León B, Martínez del Hoyo G, Parrillas V, Vargas HH, Sánchez-Mateos P, Longo N, López-Bravo M, Ardavín C. Dendritic cell differentiation potential of mouse monocytes: monocytes represent immediate precursors of CD8- and CD8+ splenic dendritic cells. *Blood*. 2004;103(7):2668-76.

Li X, Rayford H, Uhal BD. Essential roles for angiotensin receptor AT1a in bleomycin-induced apoptosis and lung fibrosis in mice. *Am J Pathol*. 2003;163(6):2523-30.

Lin SL, Castaño AP, Nowlin BT, Lupher ML Jr, Duffield JS. Bone marrow Ly6Chigh monocytes are selectively recruited to injured kidney and differentiate into functionally distinct populations. *J Immunol*. 2009;183(10):6733-43.

Liu JY, Sime PJ, Wu T, Warshamana GS, Pociask D, Tsai SY, Brody AR. Transforming growth factor-beta(1) overexpression in tumor necrosis factor-alpha receptor knockout mice induces fibroproliferative lung disease. *Am J Respir Cell Mol Biol.* 2001 Jul;25(1):3-7.

McCullough B, Collins JF, Johanson WG Jr, Grover FL. Bleomycin-induced diffuse interstitial pulmonary fibrosis in baboons. *J Clin Invest*. 1978;61(1):79-88.

McDonald S, Rubin P, Chang AY, Penney DP, Finkelstein JN, Grossberg S, Feins R, Gregory PK. Pulmonary changes induced by combined mouse beta-interferon (rMuIFN-beta) and irradiation in normal mice--toxic versus protective effects. *Radiother Oncol*. 1993;26(3):212-8.

McKnight AJ, Gordon S. The EGF-TM7 family: unusual structures at the leukocyte surface. *J Leukoc Biol*. 1998 Mar;63(3):271-80.

McNally AK, Macewan SR, Anderson JM. alpha subunit partners to beta1 and beta2 integrins during IL-4-induced foreign body giant cell formation. *J Biomed Mater Res A.* 2007;82(3):568-74.

MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol.* 1997;15:323-50.

Madtes DK, Elston AL, Kaback LA, Clark JG. Selective induction of tissue inhibitor of metalloproteinase-1 in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol.* 2001;24(5):599-607.

Maeda A, Hiyama K, Yamakido H, Ishioka S, Yamakido M. Increased expression of platelet-derived growth factor A and insulin-like growth factor-I in BAL cells during the development of bleomycin-induced pulmonary fibrosis in mice. *Chest.* 1996;109(3):780-786.

Maher TM, Wells AU. Lost in translation; from animal models of pulmonary fibrosis to human disease. *Respirology.* 2009;14(7):915-6.

Manicone AM, Birkland TP, Lin M, Betsuyaku T, van Rooijen N, Lohi J, Keski-Oja J, Wang Y, Skerrett SJ, Parks WC. Epilysin (MMP-28) restrains early macrophage recruitment in Pseudomonas aeruginosa pneumonia. *J Immunol*. 2009;182(6):3866-3876.

Mantovani A, Locati M, Vecchi A, Sozzani S, Allavena P. Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. *Trends Immunol*. 2001;22(6):328-36.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11): 549-555.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-86.

Mapel DW, Samet JM, Coultas DB. Corticosteroids and the treatment of idiopathic pulmonary fibrosis: past, present, and future. *Chest* 1996;110:1058–1067.

Margolin SB, Lefkowitz S. Pirfenidone: a novel pharmacologic agent for prevention and resolution (removal) of lung fibrosis. *FASEB J* 1994;8(4):A117.

Marriott HM, Hellewell PG, Cross SS, Ince PG, Whyte MK, Dockrell DH. Decreased alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia. *J Immunol*. 2006;177(9):6480-8.

Martinet Y, Rom WN, Grotendorst GR, Martin GR, Crystal RG. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. *N Engl J Med*. 1987;317(4):202-9.

Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol*. 2006;177(10):7303-11.

Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13:453-61.

Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol*. 2009;27:451-483.

Mathai SK, Gulati M, Peng X, Russell TR, Shaw AC, Rubinowitz AN, Murray LA, Siner JM, Antin-Ozerkis DE, Montgomery RR, Reilkoff RA, Bucala RJ, Herzog EL.. Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab Invest*. 2010 Apr 19. [Epub ahead of print]

Matute-Bello G, Winn RK, Jonas M, Chi EY, Martin TR, Liles WC. Fas (CD95) induces alveolar epithelial cell apoptosis in vivo: implications for acute pulmonary inflammation. *Am J Pathol*. 2001;158(1):153-61.

Matute-Bello G, Lee JS, Frevert CW, Liles WC, Sutlief S, Ballman K, Wong V, Selk A, Martin TR. Optimal timing to repopulation of resident alveolar macrophages with donor cells following total body irradiation and bone marrow transplantation in mice. *J Immunol Methods*. 2004;292:25–34.

Maus UA, Janzen S, Wall G, Srivastava M, Blackwell TS, Christman JW, Seeger W, Welte T, Lohmeyer J. Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. *Am J Respir Cell Mol Biol*. 2006;35(2):227-35.

Mehrad B, Burdick MD, Strieter RM. Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis. *Int J Biochem Cell Biol*. 2009;41(8-9):1708-18.

Misson P, van den Brûle S, Barbarin V, Lison D, Huaux F. Markers of macrophage differentiation in experimental silicosis. *J Leukoc Biol*. 2004;76(5):926-932.

Modolell M, Corraliza IM, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol*. 1995;25(4):1101-4.

Moeller A, Ask K, Warburton D, Gauldie J, Kolb M. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol.* 2008;40(3):362-82.

Moeller A, Gilpin SE, Ask K, Cox G, Cook D, Gauldie J, Margetts PJ, Farkas L, Dobranowski J, Boylan C, O'Byrne PM, Strieter RM, Kolb M. Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2009;179(7):588-94.

Moestrup SK, Møller HJ. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann Med*. 2004;36(5):347-354.

Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol*. 2005;166(3):675-84.

Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol.* 2008;294(2):L152-160.

Mora AL, Torres-González E, Rojas M, Corredor C, Ritzenthaler J, Xu J, Roman J, Brigham K, Stecenko A. Activation of alveolar macrophages via the alternative pathway in herpesvirus-induced lung fibrosis. *Am J Respir Cell Mol Biol*. 2006;35(4):466-473.

Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, Weaver AL, Keystone EC, Furst DE, Mease PJ, Ruderman EM, Horwitz DA, Arkfeld

DG, Garrison L, Burge DJ, Blosch CM, Lange ML, McDonnell ND, Weinblatt ME. Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. *Ann Intern Med*. 1999;130(6):478-86.

Morga E, Heuschling P. Interleukin-4 down-regulates MHC class II antigens on cultured rat astrocytes. *Glia.* 1996 Jun;17(2):175-9.

Mosser DM, Handman E. Treatment of murine macrophages with interferon-gamma inhibits their ability to bind leishmania promastigotes. *J Leukoc Biol*. 1992;52(4):369-76.

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989;7:145-73.

Mukae H, Iiboshi H, Nakazato M, Hiratsuka T, Tokojima M, Abe K, Ashitani J, Kadota J, Matsukura S, Kohno S. Raised plasma concentrations of alpha-defensins in patients with idiopathic pulmonary fibrosis. *Thorax* 2002;57:623–628.

Mueller TD, Zhang JL, Sebald W, Duschl A. Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochim Biophys Acta*. 2002;1592(3):237-50.

Mukhopadhyay S, Gordon S. The role of scavenger receptors in pathogen recognition and innate immunity. *Immunobiology*. 2004;209(1-2):39-49.

Mukhopadhyay S, Chen Y, Sankala M, Peiser L, Pikkarainen T, Kraal G, Tryggvason K, Gordon S. MARCO, an innate activation marker of macrophages, is a class A scavenger receptor for Neisseria meningitidis. *Eur J Immunol*. 2006;36(4):940-9.

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D. The integrin alpha v beta 6 binds and

activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell.* 1999;96(3):319-328.

Murray LA, Rosada R, Moreira AP, Joshi A, Kramer MS, Hesson DP, Argentieri RL, Mathai S, Gulati M, Herzog EL, Hogaboam CM. Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PLoS One*. 2010;5(3):e9683.

Nagaoka I, Trapnell BC, Crystal RG. Upregulation of platelet-derived growth factor-A and -B gene expression in alveolar macrophages of individuals with idiopathic pulmonary fibrosis. *J Clin Invest*. 1990;85(6):2023-2027.

Nagai S, Kitaichi M, Hamada K, Nagao T, Hoshino Y, Miki H, Izumi T. Hospital-based historical cohort study of 234 histologically proven Japanese patients with IPF. *Sarcoidosis Vasc Diffuse Lung Dis*. 1999; 16:209–214.

Naglich JG, Metherall JE, Russell DW, Eidels L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell*. 1992;69(6):1051-61.

Nakayama S, Mukae H, Sakamoto N, Kakugawa T, Yoshioka S, Soda H, Oku H, Urata Y, Kondo T, Kubota H, Nagata K, Kohno S. Pirfenidone inhibits the expression of HSP47 in TGF-beta1-stimulated human lung fibroblasts. *Life Sci*. 2008;82(3-4):210-7.

Nerlich A. Morphology of basement membrane and associated matrix proteins in normal and pathological tissues. *Veroff Pathol. 1*995;145:1-139.

Nicolás FJ, Lehmann K, Warne PH, Hill CS, Downward J. Epithelial to mesenchymal transition in Madin-Darby canine kidney cells is accompanied by down-regulation of

Smad3 expression, leading to resistance to transforming growth factor-beta-induced growth arrest. *J Biol Chem*. 2003;278(5):3251-6.

Niedermeier M, Reich B, Rodriguez Gomez M, Denzel A, Schmidbauer K, Göbel N, Talke Y, Schweda F, Mack M. CD4+ T cells control the differentiation of Gr1+ monocytes into fibrocytes. *Proc Natl Acad Sci U S A*. 2009;106(42):17892-7.

Oda D, Gown AM, Vande Berg JS, Stern R. The fibroblast-like nature of myofibroblasts. *Exp Mol Pathol*. 1988;49(3):316-29.

Okamoto T, Ichiyasu H, Ichikado K, Muranaka H, Sato K, Okamoto S, Iyonaga K, Suga M, Kohrogi H. [Clinical analysis of the acute exacerbation in patients with idiopathic pulmonary fibrosis]. *Nihon Kokyuki Gakkai Zasshi* 2006;44:359–367. Japanese.

Okuma T, Terasaki Y, Kaikita K, Kobayashi H, Kuziel WA, Kawasuji M, Takeya M. C-C chemokine receptor 2 (CCR2) deficiency improves bleomycin-induced pulmonary fibrosis by attenuation of both macrophage infiltration and production of macrophage-derived matrix metalloproteinases. *J Pathol.* 2004;204(5):594-604.

Olson AL, Swigris JJ, Lezotte DC, Norris JM, Wilson CG, Brown KK. Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003. *Am J Respir Crit Care Med*. 2007;176(3):277-84.

Olson AL, Huie TJ, Groshong SD, Cosgrove GP, Janssen WJ, Schwarz MI, Brown KK, Frankel SK. Acute exacerbations of fibrotic hypersensitivity pneumonitis: a case series. *Chest*. 2008;134(4):844-50.

Pardo A, Ruiz V, Arreola JL, Ramírez R, Cisneros-Lira J, Gaxiola M, Barrios R, Kala SV, Lieberman MW, Selman M. Bleomycin-induced pulmonary fibrosis is attenuated in

gamma-glutamyl transpeptidase-deficient mice. *Am J Respir Crit Care Med*. 2003;167(6):925-32.

Pardo A, Selman M. Matrix metalloproteases in aberrant fibrotic tissue remodeling. *Proc Am Thorac Soc*. 2006;3(4):383-8.

Pardo A, Selman M, Kaminski N. Approaching the degradome in idiopathic pulmonary fibrosis. *Int J Biochem Cell Biol*. 2008;40(6-7):1141-55.

Patterson ML, Atkinson SJ, Kna¨uper V, Murphy G. Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett* 2001;503:158–162.

Pérez-Ramos J, de Lourdes Segura-Valdez M, Vanda B, Selman M, Pardo A. Matrix metalloproteinases 2, 9, and 13, and tissue inhibitors of metalloproteinases 1 and 2 in experimental lung silicosis. *Am J Respir Crit Care Med*. 1999;160(4):1274-82.

Perez-Tamayo R. Morphostasis: regulation of structure. *Bol Estud Med Biol* 1974;145–161.

Peters SG, McDougall JC, Douglas WW, Coles DT, DeRemee RA. Colchicine in the treatment of pulmonary fibrosis. *Chest* 1993;103: 101–104.

Phan SH. The myofibroblast in pulmonary fibrosis. *Chest* 2002;122(6, Suppl):286S–289S.

Phan SH. Biology of fibroblasts and myofibroblasts. *Proc Am Thorac Soc*. 2008;5(3):334-7.

Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM: Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest*. 2004;114:438–46.

Plataki M, Koutsopoulos AV, Darivianaki K, Delides G, Siafakas NM, Bouros D. Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. *Chest.* 2005;127(1):266-74.

Prasse A, Pechkovsky DV, Toews GB, Jungraithmayr W, Kollert F, Goldmann T, Vollmer E, Müller-Quernheim J, Zissel G. A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18. *Am J Respir Crit Care Med.* 2006;173(7):781-792.

Prasse A, Pechkovsky DV, Toews GB, Schäfer M, Eggeling S, Ludwig C, Germann M, Kollert F, Zissel G, Müller-Quernheim J. CCL18 as an indicator of pulmonary fibrotic activity in idiopathic interstitial pneumonias and systemic sclerosis. *Arthritis Rheum.* 2007;56(5):1685-93.

Prasse A, Probst C, Bargagli E, Zissel G, Toews GB, Flaherty KR, Olschewski M, Rottoli P, Müller-Quernheim J. Serum CC-chemokine ligand 18 concentration predicts outcome in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2009;179(8):717-23.

Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R. Circulating fibrocytes: collagen-secreting cells of the peripheral blood. *Int J Biochem Cell Biol*. 2004;36(4):598-606.

Quinones F, Crouch E Biosynthesis of interstitial and basement membrane collagens in pulmonary fibrosis. *Am Rev Respir Dis.* 1986;134(6):1163-71.

Raes G, De Baetselier P, Noël W, Beschin A, Brombacher F, Hassanzadeh Gh G. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol.* 2002;71(4):597-602.

Raghu G, Striker LJ, Hudson LD, Striker GE. Extracellular matrix in normal and fibrotic human lungs. *Am Rev Respir Dis.* 1985;131(2):281-9.

Raghu G, Depaso WJ, Cain K, Hammar SP, Wetzel CE, Dreis DF, Hutchinson J, Pardee NE, Winterbauer RH. Azathioprine combined with prednisone in the treatment of idiopathic pulmonary fibrosis: a prospective, double-blind randomized, placebo-controlled clinical trial. *Am Rev Respir Dis* 1991;144:291–296.

Raghu G, Johnson WC, Lockhart D, Mageto Y. Treatment of idiopathic pulmonary fibrosis with a new antifibrotic agent, pirfenidone: results of a prospective, open-label Phase II study. *Am J Respir Crit Care Med.* 1999;159(4 Pt 1):1061-9.

Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA, King TE Jr; Idiopathic Pulmonary Fibrosis Study Group. A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 2004;350:125–133.

Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2006;174(7):810-6.

Raghu G, Brown KK, Costabel U, Cottin V, du Bois RM, Lasky JA, Thomeer M, Utz JP, Khandker RK, McDermott L, Fatenejad S. Treatment of idiopathic pulmonary

fibrosis with etanercept: an exploratory, placebo-controlled trial. *Am J Respir Crit Care Med.* 2008;178(9):948-55.

Raisfeld IH, Chovan JP, Frost S. Bleomycin pulmonary toxicity: production of fibrosis by bithiazole-terminal amine and terminal amine moieties of bleomycin A2. *Life Sci*. 1982;30(16):1391-8.

Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG. Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. *Proc Natl Acad Sci U S A.* 1981;78(11):7147-7151.

Rennard SI, Bitterman PB, Ozaki T, Rom WN, Crystal RG. Colchicine suppresses the release of fibroblast growth factors from alveolar macrophages in vitro. The basis of a possible therapeutic approach to the fibrotic disorders. *Am Rev Respir Dis.* 1988;137(1):181-185.

Richeldi L,Davies HR, Ferrara G, Franco F. Corticosteroids for idiopathic pulmonary fibrosis. Cochrane Database Syst Rev 2003;issue 3:CD002880.

Roberts SN, Howie SE, Wallace WA, Brown DM, Lamb D, Ramage EA, Donaldson K. A novel model for human interstitial lung disease: hapten-driven lung fibrosis in rodents. *J Pathol*. 1995;176(3):309-18.

Rom WN, Basset P, Fells GA, Nukiwa T, Trapnell BC, Crysal RG.Alveolar macrophages release an insulin-like growth factor I-type molecule. *J Clin Invest*. 1988;82(5):1685-93.

Rorsman F, Bywater M, Knott TJ, Scott J, Betsholtz C. Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: alternative exon usage predicts two different precursor proteins. *Mol Cell Biol*. 1988;8(2):571-7.

Rosenkilde MM, Schwartz TW. The chemokine system -- a major regulator of angiogenesis in health and disease. *APMIS*. 2004;112(7-8):481-95.

Rouhani FN, Brantly ML, Markello TC, Helip-Wooley A, O'Brien K, Hess R, Huizing M, Gahl WA, Gochuico BR. Alveolar Macrophage Dysregulation in Hermansky-Pudlak Syndrome Type-1. *Am J Respir Crit Care Med*. 2009;180(11):1114-1121.

Routes JM, Cook JL. Resistance of human cells to the adenovirus E3 effect on class I MHC antigen expression. Implications for antiviral immunity. *J Immunol*. 1990;144(7):2763-70.

Rube CE, Uthe D, Schmid KW, Richter KD, Wessel J, Schuck A, Willich N, Rube C. Dose-dependent induction of transforming growth factor beta (TGF-beta) in the lung tissue of fibrosis-prone mice after thoracic irradiation. *Int J Radiat Oncol Biol Phys*. 2000;47(4):1033-42.

Scadding JG, Hinson KF. Diffuse fibrosing alveolitis (diffuse interstitial fibrosis of the lungs). Correlation of histology at biopsy with prognosis. *Thorax*. 1967;22(4):291-304.

Schelegle ES, Mansoor JK, Giri S. Pirfenidone attenuates bleomycin-induced changes in pulmonary functions in hamsters. *Proc Soc Exp Biol Med*. 1997;216(3):392-7.

Schepers GW, Durkan TM, Delahant AB, Creedon FT, Redlin AJ. The biological action of Degussa submicron amorphous silica dust (Dow Corning silica). I. Inhalation studies on rats. *AMA Arch Ind Health*. 1957;16(2):125-46.

Schrier DJ, Kunkel RG, Phan SH. The role of strain variation in murine bleomycin-induced pulmonary fibrosis. *Am Rev Respir Dis*. 1983;127(1):63-6.

Scotton CJ, Martinez FO, Smelt MJ, Sironi M, Locati M, Mantovani A, Sozzani S. Transcriptional profiling reveals complex regulation of the monocyte IL-1 beta system by IL-13. *J Immunol*. 2005;174(2):834-45.

Scotton CJ and Chambers RC. Molecular Targets in Pulmonary Fibrosis: The Myofibroblast in Focus. *Chest* 2007; 132: 1311-21.

Sebti SM, Mignano JE, Jani JP, Srimatkandada S, Lazo JS. Bleomycin hydrolase: molecular cloning, sequencing, and biochemical studies reveal membership in the cysteine proteinase family. *Biochemistry.* 1989 Aug 8;28(16):6544-8.

Selman M, Carrillo G, Salas J, Padilla RP, Pe´rez-Chavira R, Sansores R, Chapela R. Colchicine, D-penicillamine, and prednisone in the treatment of idiopathic pulmonary fibrosis: a controlled clinical trial. *Chest* 1998;114:507–512.

Selman M, Ruiz V, Cabrera S, Segura L, Ramírez R, Barrios R, Pardo A. TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? *Am J Physiol Lung Cell Mol Physiol.* 2000;279(3):L562-74.

Selman M, King TE Jr and Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med.* 2001;134:136–51.

Selman M, Thannickal VJ, Pardo A, Zisman DA, Martinez FJ, Lynch JP 3rd. Idiopathic pulmonary fibrosis: pathogenesis and therapeutic approaches. *Drugs*. 2004;64(4):405-30

Selman M, Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc*. 2006;3(4):364-372.

Selman M, Carrillo G, Estrada A, Mejia M, Becerril C, Cisneros J, Gaxiola M, Pérez-Padilla R, Navarro C, Richards T, Dauber J, King TE Jr, Pardo A, Kaminski N. Accelerated variant of idiopathic pulmonary fibrosis: clinical behavior and gene expression pattern. *PLoS One*. 2007;2(5):e482.

Sharplin J, Franko AJ. A quantitative histological study of strain-dependent differences in the effects of irradiation on mouse lung during the early phase. *Radiat Res.* 1989;119(1):1-14.

Sheppard D. Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc*. 2006;3(5):413-417.

Shukla A, Barrett TF, Nakayama KI, Nakayama K, Mossman BT, Lounsbury KM. Transcriptional up-regulation of MMP12 and MMP13 by asbestos occurs via a PKCdelta-dependent pathway in murine lung. *FASEB J.* 2006;20(7):997-9.

Sibille Y and Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis.* 1990;141:471–501.

Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest.* 1997;100(4):768-76.

Skeen MJ, Miller MA, Shinnick TM, Ziegler HK. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. *J Immunol*. 1996;156(3):1196-206.

a. Snider GL, Hayes JA, Korthy AL. Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: pathology and stereology. *Am Rev Respir Dis.* 1978;117(6):1099-1108.

b. Snider GL, Celli BR, Goldstein RH, O'Brien JJ, Lucey EC. Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin. Lung volumes, volume-pressure relations, carbon monoxide uptake, and arterial blood gas studied. *Am Rev Respir Dis.* 1978;117(2):289-97.

Song E, Ouyang N, Hörbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol*. 2000;204(1):19-28.

Standiford TJ, Strieter RM, Chensue SW, Westwick J, Kasahara K, Kunkel SL. IL-4 inhibits the expression of IL-8 from stimulated human monocytes. *J Immunol*. 1990;145(5):1435-9.

Stefanou A, Siderov J; Society of Hospital Pharmacists of Australia Committee of Specialty Practice in Oncology. Medical errors. Dosage nomenclature of bleomycin needs to be standardised to avoid errors. *BMJ*. 2001;322(7299):1423-4.

Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*. 1992;176(1):287-92.

Stramer BM, Mori R, Martin P. The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. *J Invest Dermatol*. 2007 May;127(5):1009-17.

Strauss-Ayali D, Conrad SM and Mosser DM. Monocyte subpopulations and their differentiation patterns during infection. *J Leukoc Biol*. 2007;82(2):244-52.

Strieter RM. Con: Inflammatory mechanisms are not a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2002; 165(9):1206-7.

a. Strieter RM, Belperio JA, Burdick MD, Keane MP. CXC chemokines in angiogenesis relevant to chronic fibroproliferation. *Curr Drug Targets Inflamm Allergy*. 2005;4(1):23-6.

b. Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev*. 2005;16(6):593-609.

Sunderkötter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004 Apr 1;172(7):4410-7.

Swiderski RE, Dencoff JE, Floerchinger CS, Shapiro SD, Hunninghake GW. Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *Am J Pathol.* 1998;152(3):821-8.

Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology*. 2006;211(6-8):609-18.

Tajima S, Oshikawa K, Tominaga S, Sugiyama Y. The increase in serum soluble ST2 protein upon acute exacerbation of idiopathic pulmonary fibrosis. *Chest* 2003;124:1206–1214.

Taniguchi H, Ebina M, Kondoh Y, Ogura T, Azuma A, Suga M, Taguchi Y, Takahashi H, Nakata K, Sato A, Takeuchi M, Raghu G, Kudoh S, Nukiwa T; Pirfenidone Clinical

Study Group in Japan. Pirfenidone in idiopathic pulmonary fibrosis. *Eur Respir J.* 2010;35(4):821-9.

Tanjore H, Xu XC, Polosukhin VV, Degryse AL, Li B, Han W, Sherrill TP, Plieth D, Neilson EG, Blackwell TS, Lawson WE. Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. *Am J Respir Crit Care Med*. 2009;180(7):657-65.

Tarling JD, Lin HS and Hsu, S. Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies. *J Leukoc Biol*. 1987;42:443–6.

Taut K, Winter C, Briles DE, Paton JC, Christman JW, Maus R, Baumann R, Welte T, Maus UA. Macrophage Turnover Kinetics in the Lungs of Mice Infected with Streptococcus pneumoniae. *Am J Respir Cell Mol Biol*. 2008;38(1):105-13.

Taylor PR, Brown GD, Geldhof AB, Martinez-Pomares L, Gordon S. Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo. *Eur J Immunol*. 2003;33(8):2090-7.

Thepen T, Van Rooijen N, Kraal G. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med*. 1989;170(2):499-509.

Thrall RS, McCormick JR, Jack RM, McReynolds RA, Ward PA. Bleomycin-induced pulmonary fibrosis in the rat: inhibition by indomethacin. *Am J Pathol*. 1979;95(1):117-30.

Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc Natl Acad Sci U SA*. 2007;104(49):19446-19451.

Töröcsik D, Bárdos H, Nagy L, Adány R. Identification of factor XIII-A as a marker of alternative macrophage activation. *Cell Mol Life Sci*. 2005;62(18):2132-9.

Transplant activity in the UK 2007-2008. http://www.uktransplant.org.uk/ukt/statistics/transplant_activity_report/archive_activity_reports/pdf/ukt/transplant_activity_uk_2007-2008.pdf)

Travis WD, Hunninghake G, King TE Jr, Lynch DA, Colby TV, Galvin JR, Brown KK, Chung MP, Cordier JF, du Bois RM, Flaherty KR, Franks TJ, Hansell DM, Hartman TE, Kazerooni EA, Kim DS, Kitaichi M, Koyama T, Martinez FJ, Nagai S, Midthun DE, Müller NL, Nicholson AG, Raghu G, Selman M, Wells A. Idiopathic nonspecific interstitial pneumonia: report of an American Thoracic Society project. *Am J Respir Crit Care Med*. 2008;177(12):1338-47.

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol*. 2003;3(2):133-46.

a. Turner-Warwick M, Burrows B, Johnson A. Cryptogenic fibrosing alveolitis: clinical features and their influence on survival. *Thorax.* 1980;35(3):171-80.

b. Turner-Warwick M, Burrows B, Johnson A. Cryptogenic fibrosing alveolitis: response to corticosteroid treatment and its effect on survival. *Thorax.* 1980;35(8):593-9.

Uchinami H, Seki E, Brenner DA, D'Armiento J. Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. *Hepatology*. 2006 Aug;44(2):420-9.

Uh ST, Inoue Y, King TE Jr, Chan ED, Newman LS, Riches DW. Morphometric analysis of insulin-like growth factor-I localization in lung tissues of patients with

idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 1998;158(5 Pt 1):1626-1635.

Upreti RK, Sahu AP, Shukla L, Srivastava SN, Shanker R. Bleomycin induced changes in lungs & lymph nodes of guineapigs. *Indian J Exp Biol*. 1979 Sep;17(9):922-5.

Uría JA, Jiménez MG, Balbín M, Freije JM, López-Otín C. Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J Biol Chem.* 1998;273(16):9769-77.

Usuki, KFY. Evolution of three patterns of intra-alveolar fibrosis produced by bleomycin in rats. *Pathol Int.* 1995;45:552–64.

van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med*. 1968;128(3):415-35.

van Furth R, Hirsch JG, Fedorko ME. Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes, and macrophages. *J Exp Med*. 1970;132(4):794-812.

Van Furth R, Diesselhoff-den Dulk MC, Mattie H. Quantitative study on the production and kinetics of mononuclear phagocytes during an acute inflammatory reaction. *J Exp Med*. 1973;138(6):1314-30.

van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med*. 2005;201(6):981-991.

van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174(1-2):83-93.

van Rooijen N, Sanders A and van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol* Methods 1996; 193(1): 93-9.

Valledor AF, Ricote M. Nuclear receptor signaling in macrophages. *Biochem Pharmacol.* 2004;67(2):201-12.

Varin A, Gordon S. Alternative activation of macrophages: Immune function and cellular biology. *Immunobiology.* 2009 Mar 3. [Epub ahead of print].

Veeraraghavan S, Latsi PI, Wells AU, Pantelidis P, Nicholson AG, Colby TV, Haslam PL, Renzoni EA, du Bois RM. BAL findings in idiopathic nonspecific interstitial pneumonia and usual interstitial pneumonia. *Eur Respir J*. 2003;22(2):239-44.

Walter N, Collard HR, King TE Jr. Current perspectives on the treatment of idiopathic pulmonary fibrosis. *Proc Am Thorac Soc*. 2006;3(4):330-8.

Walters DM, Cho HY, Kleeberger SR. Oxidative stress and antioxidants in the pathogenesis of pulmonary fibrosis: a potential role for Nrf2. *Antioxid Redox Signal*. 2008;10(2):321-332.

Wang J, Roderiquez G, Oravecz T, Norcross MA. Cytokine regulation of human immunodeficiency virus type 1 entry and replication in human monocytes/macrophages through modulation of CCR5 expression. *J Virol*. 1998;72(9):7642-7.

Wang Q, Wang Y, Hyde DM, Gotwals PJ, Koteliansky VE, Ryan ST, Giri SN. Reduction of bleomycin induced lung fibrosis by transforming growth factor beta soluble receptor in hamsters. *Thorax*. 1999;54(9):805-12.

Wang R, Ibarra-Sunga O, Verlinski L, Pick R, Uhal BD. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am J Physiol Lung Cell Mol Physiol.* 2000 Jul;279(1):L143-51.

Wang L, Antonini JM, Rojanasakul Y, Castranova V, Scabilloni JF, Mercer RR. Potential Role of Apoptotic Macrophages in Pulmonary Inflammation and Fibrosis. *J Cell Phys*. 2003;194(2):215–224.

Wang L, Scabilloni JF, Antonini JM, Rojanasakul Y, Castranova V, Mercer RR. Induction of secondary apoptosis, inflammation, and lung fibrosis after intratracheal instillation of apoptotic cells in rats. *Am J Physiol Lung Cell Mol Physiol*. 2006;290(4): L695–L702.

Warshamana GS, Martinez S, Lasky JA, Corti M, Brody AR. Dexamethasone activates expression of the PDGF-alpha receptor and induces lung fibroblast proliferation. *Am J Physiol*. 1998;274(4 Pt 1):L499-507.

Warshamana GS, Pociask DA, Fisher KJ, Liu JY, Sime PJ, Brody AR. Titration of non-replicating adenovirus as a vector for transducing active TGF-beta1 gene expression causing inflammation and fibrogenesis in the lungs of C57BL/6 mice. *Int J Exp Pathol*. 2002;83(4):183-201.

Warshamana GS, Pociask DA, Sime P, Schwartz DA, Brody AR. Susceptibility to asbestos-induced and transforming growth factor-beta1-induced fibroproliferative lung disease in two strains of mice. *Am J Respir Cell Mol Biol.* 2002 Dec;27(6):705-13.

Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-□1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol.* 2005;166:1321–32.

Winterbauer RH, Hammar SP, Hallman KO, Hays JE, Pardee NE, Morgan EH, Allen JD, Moores KD, Bush W, Walker JH. Diffuse interstitial pneumonitis: clinicopathologic correlations in 20 patients treated with prednizone/azathioprine. *Am J Med* 1978;65:661–672.

Wynes MW, Riches DW. Induction of macrophage insulin-like growth factor-I expression by the Th2 cytokines IL-4 and IL-13. *J Immunol*. 2003;171(7):3550-9.

Wynes MW, Frankel SK, Riches DW. IL-4-induced macrophage-derived IGF-I protects myofibroblasts from apoptosis following growth factor withdrawal. *J Leukoc Biol*. 2004;76(5):1019-27.

Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol.* 2004;4(8):583-594.

Yokoyama A, Kohno N, Hamada H, Sakatani M, Ueda E, Kondo K, Hirasawa Y, Hiwada K. Circulating KL-6 predicts the outcome of rapidly progressive idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1998;158:1680–1684.

Zhang K, Rekhter MD, Gordon D, Phan SH. Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. *Am J Pathol*. 1994;145:114–25.

Zhang Y, Feng X, We R, Derynck R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature.* 1996;383(6596):168-72.

Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest*. 2009;119(7):1899-1909.

Zhang-Hoover J, Sutton A, van Rooijen N, Stein-Streilein J. A critical role for alveolar macrophages in elicitation of pulmonary immune fibrosis. *Immunology.* 2000;101(4):501-511.

Zhao J, Shi W, Wang YL, Chen H, Bringas P Jr, Datto MB, Frederick JP, Wang XF, Warburton D. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol*. 2002;282(3):L585-93.

Zheng M, Cai WM, Zhao JK, Zhu SM, Liu RH. Determination of serum levels of YKL-40 and hyaluronic acid in patients with hepatic fibrosis due to schistosomiasis japonica and appraisal of their clinical value. *Acta Trop*. 2005 Nov-Dec;96(2-3):148-52.

Ziegenhagen MW, Zabel P, Zissel G, Schlaak M, Muller-Quernheim J. Serum level of interleukin 8 is elevated in idiopathic pulmonary fibrosis and indicates disease activity. *Am J Respir Crit Care Med* 1998;157:762–768.

Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B, Sheppard D, Pardo A, Selman M, Heller RA. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. *Proc Natl Acad Sci U S A.* 2002;99(9):6292-7.